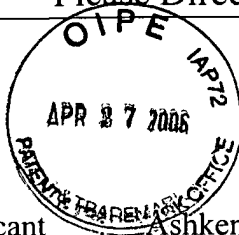


Please Direct All Correspondence to Customer Number 30313

TRANSMITTAL LETTER
APPEAL BRIEF

Applicant : Ashkenazi, et al.

App. No : 10/066,273

Filed : February 1, 2002

For : SECRETED AND
TRANSMEMBRANE POLYPEPTIDES
AND NUCLEIC ACIDS ENCODING
THE SAME

Examiner : Chernyshev, Olga N.

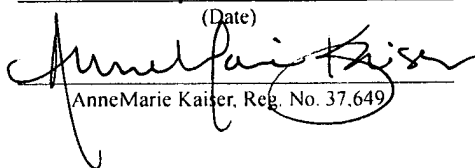
Art Unit : 1649

CERTIFICATE OF MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on

April 24, 2006

(Date)


AnneMarie Kaiser, Reg. No. 37,649

Mail Stop Appeal Brief - Patents

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

Transmitted herewith for filing in the above-identified application are the following enclosures:

- (X) Appeal Brief in forty-five (45) pages.
- (X) A copy of evidence cited in Appellant's Brief and listed in Appendix B.

FILING FEES:

FEE CALCULATION				
FEE TYPE		FEE CODE	CALCULATION	TOTAL
Appeal Brief	41.20(b)(2)	1402 (\$500)		\$500
			TOTAL FEE DUE	\$500

- (X) A check in the amount of \$500 is enclosed.
- (X) Return prepaid postcard.

Docket No. : GNE.3130R1C2
Application No. : 10/066,273
Filing Date : February 1, 2002



Customer No.: 30,313

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Dated: April 24, 2006

A handwritten signature in cursive script, reading "AnneMarie Kaiser". The signature is written over a horizontal line.

AnneMarie Kaiser
Registration No. 37,649
Attorney of Record
Customer No. 30,313
(619) 235-8550

2543371 SMA042106

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

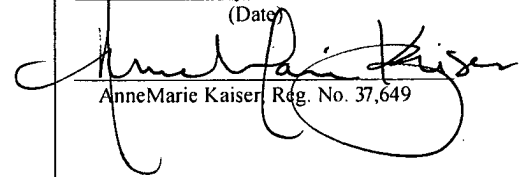
Applicant : Ashkenazi, et al.
Appl. No. : 10/066,273
Filed : February 1, 2002
For : SECRETED AND
TRANSMEMBRANE
POLYPEPTIDES AND NUCLEIC
ACIDS ENCODING THE SAME
Examiner : Chernyshev, Olga N.
Group Art Unit : 1649

CERTIFICATE OF MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on

April 24, 2006

(Date)


AnneMarie Kaiser Reg. No. 37,649

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPELLANT'S BRIEF

Mail Stop Appeal Brief – Patents
COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The Applicants appeal the rejection of Claims 40-44 in the above-captioned patent application. These claims were rejected in a final Office Action mailed November 25, 2005. Applicants filed a Notice of Appeal February 24, 2006.

I. REAL PARTY IN INTEREST

Pursuant to 37 C.F.R. 41.37(c)(1), Appellants hereby notify the Board of Patent Appeals and Interferences that the real party in interest is the assignee of record for this application, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080.

II. RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any other related appeals or interferences.

Appl. No. : **10/066,273**
Filed : **February 1, 2002**

III. STATUS OF THE CLAIMS

The above-captioned application was filed with Claims 1-39. Appellants cancelled Claims 1-39 and added new Claims 40-45 in a Preliminary Amendment⁰ mailed February 1, 2001. Appellants canceled Claim 45 in an Amendment and Response mailed July 27, 2004. Appellants amended Claim 40 in an Amendment and Response filed September 1, 2004. The Examiner rejected Claims 40-44 in a final Office Action dated November 25, 2005.

Accordingly, Claims 40-44 are the subject of this appeal. The claims attached hereto as Appendix A reflect the claims as amended by the Amendment filed with the Amendment and Response mailed September 1, 2004.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the issuance of the final Office Action dated January 24, 2006.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The claimed subject matter relates to antibodies that specifically bind to the polypeptide of SEQ ID NO: 9.

Various aspects of the claimed antibodies are described in the specification at, for example, p. 90, l. 30 through p. 99, l. 6, and Figure 4. SEQ ID NO:9 is disclosed in the Sequence Listing appended to the application.

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL AND GROUPING OF CLAIMS

A. Grounds of Rejection on Appeal

The Examiner has rejected pending Claims 40-44 under 35 U.S.C. §101, stating that the claimed invention is drawn to an invention with no apparent or disclosed specific and substantial credible utility. *Final Office Action* at 2.

The Examiner also has rejected pending Claims 40-44 under 35 U.S.C. §112, first paragraph as lacking an enabling disclosure, asserting that "since the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility ... one

skilled in the art clearly would not know how to use the claimed invention.” *Final Office Action* at 8-9.

B. Grouping of Claims

1. Utility Rejection – Claims 40-44

For purposes of the utility rejection under 35 U.S.C. § 101, Claims 40-44 can be considered as a group.

2. Enablement Rejection – Claims 40-44

For purposes of the enablement rejection under 35 U.S.C. § 112, first paragraph, Claims 40-44 can be considered as a group.

VII. APPELLANTS’ ARGUMENT

A. Summary of the Arguments

1. Utility Rejection

The first issue before the Board is whether Appellants have asserted at least one “specific, substantial, and credible utility” for the claimed subject matter. *See*, Examination Guidelines, 66 Fed. Reg. 1092 (2001). Briefly stated, Appellants’ asserted utility is based on the disclosure in Example 60 of the instant application that the PRO444 polypeptide stimulates *c-fos* expression in retinal pericyte cells. It is well-established that pericyte cells are involved in various stages of angiogenesis, including in the formation of new capillary sprouts, in promoting the survival of newly formed vasculature, and in regulating capillary permeability. Further, it is well established that pericytes secrete VEGF, a well-known potent angiogenic factor, known to be involved in cellular proliferation, survival of newly formed vasculature, and in regulating vascular permeability. Finally, it is known that *c-fos* is a component of the AP-1 transcription factor, and that AP-1 regulates expression of VEGF. The induction of *c-fos* in pericyte cells stimulates VEGF, tying *c-fos* induction in pericyte cells to angiogenesis. Accordingly, PRO444 polypeptides, which Applicants have demonstrated stimulate *c-fos* in pericyte cells, are useful as both therapeutic targets for pathological angiogenesis (*e.g.*, pericyte-associated tumors) and as

Appl. No. : 10/066,273
Filed : February 1, 2002

stimulators of angiogenesis. The claimed antibodies, which specifically bind PRO444 polypeptides, are therefore useful as therapeutic agents. The asserted utilities are specific, substantial, and credible.

2. Enablement Rejection

The second issue before the Board is whether Appellants have enabled the pending claims such that one of skill in the art would be able to make and use the claimed invention. The Examiner has rejected pending Claims 40-44 under 35 U.S.C. §112, first paragraph, arguing that because the claimed antibodies are not supported by either a specific or substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention. *Final Office Action* at 9.

Appellants submit that Claims 40-44 are enabled such that one of skill in the art could make and use the claimed antibodies without undue experimentation. Applicants submit that the claimed antibodies have a substantial, specific, and credible utility. Since the enablement rejection is based on the rejection of the claims as lacking utility, the claimed antibodies do not lack enablement.

B. Utility Rejection – Detailed Arguments

The first issue before the Board is whether Appellants have asserted at least one “specific, substantial, and credible utility” for the claimed subject matter. *See Examination Guidelines*, 66 Fed. Reg. 1092 (2001). The Examiner has rejected Claims 40-44 under 35 U.S.C. §§ 101 as lacking utility. Appellants have asserted that the claimed antibodies that specifically bind to the polypeptide of SEQ ID NO:9 (the PRO44 polypeptide) are useful as therapeutic targets for pathological angiogenesis or as tools for stimulating angiogenesis. This asserted utility is specific, substantial, and credible, as is explained in more detail below.

1. Utility – Legal Standard

A “specific utility” is defined as utility which is “specific to the subject matter claimed,” in contrast to “a general utility that would be applicable to the broad class of the invention.” *See M.P.E.P.* § 2107.01 I. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic tool without also identifying the condition that is to be diagnosed.

Appl. No. : 10/066,273
Filed : February 1, 2002

The requirement of “substantial utility” defines a “real world” use, and derives from the Supreme Court’s holding in *Brenner v. Manson*, 383 U.S. 519, 534 (1966) stating that “[t]he basic *quid pro quo* contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility.” In explaining the “substantial utility” standard, M.P.E.P. § 2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase “immediate benefit to the public” or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be “currently available” to the public in order to satisfy the utility requirement. “Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a ‘substantial’ utility.” *M.P.E.P.* § 2107.01 (emphasis added).

Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement, set forth in M.P.E.P. § 2107 II(B)(1) gives the following instruction to patent examiners: “If the applicant has asserted that the claimed invention is useful for any particular practical purpose ... and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.”

Finally, in assessing the credibility of the asserted utility, the M.P.E.P. states that “to overcome the presumption of truth that an assertion of utility by the applicant enjoys” the PTO must establish that it is “more likely than not that one of ordinary skill in the art would doubt (i.e., ‘question’) the truth of the statement of utility.” *M.P.E.P.* § 2107.02 III A.

2. Utility – Burden of Proof

It is well established that a specification which contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented “must be taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject matter unless there is reason for one skilled in the art to question the objective truth of the statement of utility or its scope.” *In re Langer*, 503 F.2d 1380, 1391, 183 U.S.P.Q. 288, 297 (C.C.P.A. 1974). Thus “the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure.” *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility

Appl. No. : 10/066,273
Filed : February 1, 2002

does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *Id.*

3. Utility – Standard of Proof

Compliance with 35 U.S.C. § 101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. 592, 596 (Fed. Cir. 1983). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or “more likely than not” standard. *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). This is stated explicitly in the M.P.E.P.:

[T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true “beyond a reasonable doubt.” Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. *M.P.E.P.* § 2107.02, part VII (emphasis in original, citations omitted).

The Court of Appeals for the Federal Circuit has stated that the standard for satisfying the utility requirement is a low one:

The threshold of utility is not high: An invention is “useful” under section 101 if it is capable of providing some identifiable benefit. *See Brenner v. Manson*, 383 U.S. 519, 534, 86 S.Ct. 1033, 16 L.Ed.2d 69 (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 (Fed. Cir. 1992) (“To violate § 101 the claimed device must be totally incapable of achieving a useful result”); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention “is incapable of serving any beneficial end”). *Juicy Whip, Inc. v. Orange Bang, Inc.*, 185 F.3d 1364, 1366, 51 U.S.P.Q. 2d 1700 (Fed. Cir. 1999) (emphasis added).

The low threshold for satisfying the utility requirement is reflected in the standard set by the Federal Circuit for invalidating a patent based on a lack of utility: “[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility. Some degree of utility is sufficient for patentability. Further, the defense of non-utility cannot be sustained without proof of total incapacity.” *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 U.S.P.Q. 473 (Fed. Cir. 1984) (emphasis added, citations omitted).

Because the standard for satisfying the utility requirement is so low, requiring total incapacity for a finding of no utility, the M.P.E.P. cautions that:

Rejections under 35 U.S.C. 101 have been *rarely* sustained by federal courts. Generally speaking, in these *rare* cases, the 35 U.S.C. 101 rejection was sustained [] because the applicant ... asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art. *M.P.E.P.* § 2107.02 III B., citing *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (C.C.P.A. 1967) (underline emphasis in original, italic emphasis added).

4. *Appellants Asserted a Specific, Substantial and Credible Utility that is Sufficient to Satisfy the Utility Requirement of § 101*

The claimed subject matter is directed to antibodies that specifically bind to the polypeptide of SEQ ID NO:9. The polypeptide of SEQ ID NO:9 (referred to as "PRO444 polypeptide") is encoded by the polynucleotide of SEQ ID NO:8 (also referred to as DNA26846-1397). *Specification*, Example 5. Appellants have asserted that the claimed antibodies are useful in the purification of PRO444 polypeptides, which in turn have utility as both therapeutic targets for tumors associated with pericytes and as stimulators of angiogenesis.

In "Example 60: Pericyte c-Fos Induction" Appellants disclose that the PRO444 polypeptide induces the expression of *c-fos* in pericyte cells at least two fold above a control polypeptide. The specification teaches that induction of *c-fos* expression in pericytes renders the polypeptides "useful. . . as giving rise to antagonists which would be expected to be useful for the therapeutic treatment of pericyte-associated tumors. . . and for the treatment of conditions where induced angiogenesis would be beneficial, including for example, wound healing, and the like." *Specification* at p. 142, lines 21-27. Example 43 describes purification of PRO polypeptides (e.g., PRO444) using specific antibodies, such as the claimed antibodies. *Specification* at p. 132-133. The specification states that PRO antibodies "can be administered for the treatment of various disorders in the form of pharmaceutical compositions." *Specification* at p. 97.

Taken together, the specification clearly discloses the use of the claimed antibodies for the purification of PRO444, which has uses both in the isolation of antagonists useful in the treatment of certain tumors, and as a stimulator of angiogenesis where angiogenesis is desirable. These utilities are specific and substantial, as one of skill in the art will recognize that the treatment of certain tumors and the stimulation of angiogenesis are not utilities that apply to the broad class of antibodies; and it is credible, as they not utilities "that could only be true if it violated a scientific principle, ...or a law of nature, or [is] wholly inconsistent with contemporary

knowledge in the art.” M.P.E.P. § 2107.02 III B., citing *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (C.C.P.A. 1967).

Because Appellants’ specification contains a disclosure of utility which corresponds in scope to the claimed subject matter, the asserted utility “must be taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject matter unless there is reason for one skilled in the art to question the objective truth of the statement of utility or its scope.” *In re Langer*, 503 F.2d 1380, 1391, 183 U.S.P.Q. 288, 297 (C.C.P.A. 1974). Therefore, the burden of establishing a *prima facie* case of lack of utility rests with the PTO. See, *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995) (“the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure”).

5. The Examiner’s Arguments

In the first Office Action, mailed April 28, 2004, the Examiner rejected the pending claims, stating “Claims 40-45 are rejected under 35 U.S.C. 101 because the claimed invention is drawn to an invention with no apparent or disclosed specific and substantial credible utility.” *Office Action* mailed April 28, 2004 at 2. This rejection is maintained in the Office Actions mailed September 17, 2004, March 16, 2005, and July 21, 2005, in the final Office Action mailed November 25, 2005, and in the Advisory Action mailed February 7, 2006. See, *Final Office Action* at 2.

To establish a *prima facie* showing that the claimed subject matter lacks utility, the Examiner must “provide[] evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility.” *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The Examiner has issued a total of five Office Actions during the prosecution of the instant application. In these Actions, the Examiner has cited a total of nine references in attempting to provide evidence that one of ordinary skill in the art would reasonably doubt Appellants’ asserted utility. As discussed below, not one of these references provides evidence that one of ordinary skill in the art would reasonably doubt the asserted utilities, and therefore does not establish the requisite *prima facie* showing to support a rejection under 35 U.S.C. § 101.

The Examiner states that the specification discloses that the PRO444 polypeptide acts to induce the expression of *c-fos* in pericyte cells, and that Appellants have asserted the use of the PRO444 polypeptides for giving rise to antagonists which would be expected to be useful for the

therapeutic treatment of tumors and for the treatment of conditions where induced angiogenesis would be beneficial. However, the Examiner rejects these utilities, arguing that “the evidence presented. . . is inadequate to support a conclusion that PRO444 induced activation of expression of *c-fos* in pericytes is specifically related to angiogenesis.” *Final Office Action* at 4.

First, the Examiner argues that many growth factors and signals activate *c-fos*, and that the role of *c-fos* is not limited to cancer. Therefore, the Examiner argues that “there appears to be no specific biological function that could be particularly attributed to PRO444 with respect to its ability to activate *c-fos* expression in pericytes.” *Office Action* mailed March 16, 2005, at 5 (emphasis added). Six of the nine references cited by the Examiner, Janknecht et al. ((1995) *Carcinogenesis*, 16(3): 443-450), Herrera et al. ((1996) *Prog. Neurobiol.* 50: 83-107), Kovács et al. ((1998) *Neurochem. Int.* 33: 287-297), Coulon et al. ((1999) *J. Biol. Chem.* 274(3): 30439-304346), Sakurai et al. ((2002) *Invest. Opth. Vis. Sci.* 43(6): 2774-2781) and Otani et al. ((2000) *Opth. Vis. Sci.* 41(5): 1192-1199), were cited to support the Examiner’s position that a specific biological function cannot be attributed a compound that induces *c-fos* in pericyte cells, and that as such PRO444 is not useful under 35 U.S.C. § 101. As discussed below, four of these six references that allegedly demonstrate that Appellants’ asserted utilities are not specific to PRO444 do not even concern *c-fos* induction in pericyte cells. Accordingly, these references are irrelevant to Appellants’ asserted utilities, which are based on induction of *c-fos* in pericyte cells, and carry no evidentiary weight in support of the Examiner’s *prima facie* showing. The remaining two references, Sakurai et al. and Otani et al., relate to *c-fos* induction in pericyte cells. As discussed below, the two relevant references provide strong support for Appellants’ asserted utilities.

Second, the Examiner argues that there is “no information at the time of filing regarding pericytes’ specific role in angiogenesis.” *See, e.g., Final Office Action* at 5. The Examiner takes the position that the role of pericytes in angiogenesis is “controversial” and that it is “presently not known whether stimulation of pericytes results in up-regulation or down-regulation of vascularization.” *See, Final Office Action* at 5; *Office Action* mailed July 21, 2005 at 3. The Examiner relies on Diaz-Florez et al. ((1994) *Histol. Histopath.* 9: 807-843) and Ozerdem et al. ((2003) *Angiogenesis* 6:241-249) in support of her position. As discussed below, both Diaz-Florez et al. and Ozerdem et al. demonstrate that pericytes have known, specific roles in angiogenesis. Contrary to the Examiner’s assertions, Diaz-Florez et al. enumerates specific

stages in angiogenesis in which it had been previously shown that pericytes play integral roles. Ozerdem et al. not only teaches that pericyte cells are involved in angiogenic sprout formation and migration, but also discloses that pericyte cells are useful for the same asserted utilities asserted by Appellants, namely as “targets for therapeutics for pathological vascularization (e.g., cancer), or as tools to facilitate vascularization (e.g., ischemic disorders).” Ozerdem et al. at 248.

Finally, the Examiner argues that the relationship between *c-fos*, AP-1 and VEGF expression is “not obvious” and that “there is no evidence that induction of. . . *c fos*. . . leads to stimulation of VEGF by means of. . . AP-1.” *Final Office Action* at 5. The Examiner relies on only one reference, Orlandini et al. ((1996) *Proc. Nat. Acad. Sci. USA* 93: 11675-11680), in support of her position. As discussed below, Orlandini et al. concerns *in vitro* experiments done in fibroblast cells. Orlandini et al. is not probative of *c-fos* regulation in pericytes. Further, the authors themselves caution readers that the reported results are inconsistent with *in vivo* studies that demonstrate *c-fos* regulation of VEGF in association with tumors.

Based on these arguments and cited references, the Examiner argues that “the instant polypeptide PRO444 is suitable only for additional research to identify or reasonably confirm a ‘real world’ context of use.” *Final Office Action* at 8. The Examiner thus concludes that PRO444 and antibodies that bind PRO444 do not have a substantial or well-established utility. *Id.*

6. **The Examiner has not established a Prima Facie case that Claims 40-44 lack Utility**

The above arguments do not satisfy the Examiner’s burden to “provide[] evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility.” *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The Examiner has the burden of presenting “countervailing facts and reasoning sufficient to establish that a person of ordinary skill would not believe the applicant’s assertion of utility.” *M.P.E.P.* at §2107.02 III.A., *citing in re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995) (“Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence”) (emphasis added). The Examiner relies on the Herrera et al., Kovács et al., Janknecht et al., Coulon et al., Sakurai et al., Otani et al., Diaz-Florez et al., Ozerdem et al., and Orlandini et al. references to

support her arguments. However, for the reasons discussed below, they do not support the Examiner's position. Therefore, the Examiner's assertions are not supported by any relevant facts, evidence, or reasoning, and there is simply no evidence in the record to support the Examiner's arguments that Appellants' asserted utility is not specific or substantial, and that the invention is incomplete. Absent some relevant evidence to support her assertions, the Examiner has failed to establish a *prima facie* showing that one of skill in the art would reasonably doubt the asserted utility, and the Board should accept Appellants' disclosed utility as sufficient.

a. *The data in Example 60 are sufficient to establish a specific asserted utility*

Appellants turn first to the Examiner's arguments that Example 60 does not support a specific utility for PRO444 polypeptides and the claimed antibodies. According to the Examiner, Appellants' evidence of *c-fos* induction in pericytes does not establish "a specific biological role for these protein [*sic*] . . . or their significance to a particular disease, disorder or physiological process, which one would wish to manipulate for a desired effect," even in light of Appellants' assertion that this activity renders PRO444 useful for treating pericyte-associated tumors or stimulating angiogenesis. *Office Action* mailed April 28, 2004 at 3. According to the Examiner "the art clearly recognizes that induction of *c-fos* expression represents a general non-specific first line of cellular response to a variety of stimuli in a variety of cells [and thus] one skilled in the art would not attribute the induction of *c-fos* in pericytes by the instant polypeptides as a physiological reaction specifically induced by these particular polypeptides." *Office Action* mailed September 17, 2004 at 4. *See also, Final Office Action* at 5 (stating that "*c-fos*. . . is known to be induced by many cellular stimuli, including growth factors, cytokines, T-cell activators, UV irradiation, hypoxia and PMA.")

In the section entitled "Specific and Substantial Requirements" for utility of inventions, the M.P.E.P. states that a specific utility "is specific to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention." M.P.E.P. §2107.02 II(A). The M.P.E.P. instructs Examiners that where an applicant discloses a particular biological activity and reasonably correlates that activity to a disease or condition, the applicant has identified a specific utility for the invention. *Id.* Appellants' specification contains an assertion of utility that meets both of the requirements. Appellants' specification states that

“induction of *c-fos* in pericytes is [] indicative of the induction of angiogenesis,” and states that this particular biological activity of PRO444 renders those peptides useful as both therapeutic targets for pathological angiogenesis and as stimulators of angiogenesis. *See, Specification*, p. 142, lines 23-25. Accordingly, Appellants have identified a particular biological activity of a compound and explained how that activity can be utilized in a particular therapeutic application of the compound, fulfilling the requirements for assertion of a specific and substantial utility for the claimed invention.

In an attempt to clarify to the Examiner that Assay 93 in Example 60 identified compounds with specific activities, Appellants submitted a declaration by Mary Gerritsen, Ph.D. with a Request for Continued Examination on January 18, 2005. Dr. Gerritsen explains that both positive and negative controls were run in Example 60 (Assay 93). Of the 648 samples assayed, only 48 samples, many of which were different lots of the same compound, tested positive for induction of *c-fos* activation in pericytes in Example 60 (Assay 93). Gerritsen Decl., ¶ 10. Dr. Gerritsen’s testimony thus establishes that *c-fos* induction in pericytes is not an activity that applies to proteins in general, and likewise that antibodies that bind proteins that induce *c-fos* cannot be a *general* utility that is applicable to the broad class of the invention.

The fact that PRO444 may not be the only factor capable of stimulating *c-fos* does not detract from Appellants’ asserted utilities. The Examiner has failed to offer sound reasoning or logic why, based on the fact that various stimuli are known to induce *c-fos*, the skilled artisan would not believe Appellants’ that PRO444 induction of *c-fos* in pericyte cells is not a specific biological activity. The Examiner has also failed to offer sound reasoning or logic why the fact that *c-fos* can be induced in neuronal cells or other cells, the skilled artisan would not believe that *c-fos* induction in pericyte cells (specialized cells known to be involved in angiogenesis) is indicative of angiogenesis.

In summary, Appellants’ asserted utilities for the claimed antibodies are not “a *general* utility that is applicable to the broad class of the invention.” M.P.E.P. §2107.02 II(A). Appellants have described a “specific” utility for the claimed antibodies.

- b. *The evidence cited by the Examiner does not refute Appellants’ assertion that induction of c-fos in pericytes is a physiological reaction specifically induced by PRO444*

Appl. No. : 10/066,273
Filed : February 1, 2002

Appellants next turn to the evidence cited by the Examiner that demonstrates that *c-fos* is induced by several stimuli, which the Examiner argues establishes that PRO444 polypeptides lack a biological function that could be particularly attributed to PRO444. Appellants discuss below each of the references relied upon by the Examiner in support of her position.

The Examiner argues that Janknecht et al. demonstrates that “the *c-fos* protooncogene is a member of the immediate-early genes (IEGs), which are rapidly induced upon stimulation of cells with growth factors, cytokines, serum, or UV light.” *Office Action* mailed April 24, 2004 at 3. Janknecht et al. is a general review of the regulatory elements at the *c-fos* promoter. Inasmuch as the teachings of Janknecht et al. are relevant to *c-fos* regulation in any particular cell type, (e.g., pericyte cells), Appellants maintain that the teachings of Janknecht et al. provide strong support for Appellants’ asserted utilities. First, Janknecht et al. teaches that several of the “various stimuli” for *c-fos* noted by the Examiner that induce *c-fos* do so through the Raf-1 pathway. Janknecht et al. at 445. As discussed below, at the time of filing it was known that VEGF was also activated through the Raf-1 pathway, thereby linking many of the “various stimuli” that induce *c-fos* to the induction of VEGF expression, a well-established angiogenic factor. See, Kolch et al. (1995) *Breast Cancer Res. Treat.* 36:139-155 at 144-145. Janknecht et al. also teaches that *c-fos* is regulated by the cAMP pathway. cAMP is also known regulator of VEGF expression. See, Kolch et al. at 141; Sakurai et al. at 2780. Finally, Janknecht et al. teaches that in 1995 it was well known that *c-fos* regulates cellular proliferation and differentiation. Janknecht et al. at 443. Appellants assertion that *c-fos* stimulation in pericytes is associated with angiogenesis, which involves both cellular proliferation and differentiation, is fully consistent with the teachings of Janknecht et al. In other words, rather than providing any evidence that would lead the skilled artisan to doubt Appellants’ asserted utilities, the teachings of Janknecht et al. weigh strongly in favor of Appellants’ asserted utilities.

In a similar line of reasoning, the Examiner cites to Herrera et al. and Kovács et al. as demonstrating that the disclosure in Example 60 does not provide evidence that PRO444 has a particular, specific activity. See, *Office Action* mailed September 17, 2004 at 3-4; *Office Action* mailed April 24, 2004 at 4. According to the Examiner, Herrera et al. and Kovács et al. teach that *c-fos* is induced by neurotropic factors, neurotransmitters, depolarizing agents, or ion channel activating agents. *Office Action* mailed April 24, 2004 at 4.

Herrera et al. is a review article entitled "Activation of *c-fos* in the Brain." Herrera et al. does not touch on the subject of *c-fos* activation in pericytes and consequently has no bearing on Appellants' asserted utilities. Interestingly, however, Herrera et al. mentions that studies on *c-fos* activation following brain wounds have led scientists to propose a role for *c-fos* in wound healing in the brain. Herrera et al. at 90. As such, inasmuch as Herrera et al. provides any evidence probative of Appellants' asserted utilities, Herrera et al. weighs in favor of Appellants' assertion that PRO444 polypeptides are useful in inducing angiogenesis, for example, in wound healing.

Kovács et al. is yet another review article that describes the role of *c-fos* as a functional marker of activated neurons. Like Herrera et al., Kovács et al. does not touch on the subject of *c-fos* activation in pericytes and consequently has no bearing on Appellants' asserted utilities.

In short, Herrera et al. and Kovács et al. are not relevant to Appellants' asserted utilities and certainly are not sufficient to establish a *prima facie* case of lack of utility under 35 U.S.C. § 101.

The Examiner has also relied upon Coulon et al. for the same proposition described above, *i.e.*, "that induction of *c-fos* can be evoked by a variety of extracellular stimuli." *Office Action* mailed March 16, 2005 at 5. Further, the Examiner asserts that "with respect to the issue of activation of *c-fos* and cell specificity" Coulon et al. "clearly indicate that not only nervous cells but cells of different types response [*sic*] to 'wide range of extracellular stimuli' by activation of immediate early response gene *c-fos*," allegedly demonstrating that *c-fos* induction in pericytes is not associated with a specific or substantial utility. *Office Action* mailed March 16, 2005 at 6. The Examiner argues that these facts show that the disclosure in Example 60 is insufficient to demonstrate that PRO444 has a specific biological function. Coulon et al. is a study conducted on mouse Ltk⁻ fibroblast cells. Coulon et al. showed that "calcium ionophore acts in synergy with either cAMP or PMA to strongly induce the endogenous *c-fos* gene." Coulon et al., abstract. As discussed above, Appellants' asserted utilities are based on experiments performed in pericyte cells, specialized cells known to play specific roles in angiogenesis. Coulon et al. concerns a different cell type, *i.e.*, mouse Ltk⁻ fibroblast cells. As such, Coulon et al. is not probative of Appellants' asserted utilities. Moreover, cAMP and PMA are well known inducers of VEGF, a potent angiogenic factor. *See*, Kolch et al. at 141;

Balabanov et al. at 640, and references cited therein. Thus, even if Coulon et al. was relevant, the teachings of Coulon et al. are fully consistent with and support Appellants' asserted utilities.

Appellants next turn to the remaining two references relied upon by the Examiner for the proposition that the activity of PRO444 does not support a specific (and presumably substantial) utility, Sakurai et al. and Otani et al. Unlike the evidence discussed above, both Sakurai et al. and Otani et al. concern *c-fos* induction in pericyte cells.

The Examiner argues that Sakurai et al. teaches that *c-fos* mRNA is induced in pericyte cells by fetal calf serum (FCS) and various prostaglandins, (*i.e.*, PDG₂) and thus shows that "activation of *c-fos* is a non-specific immediate cellular response to plurality [*sic*] of different factors." *Office Action* mailed November 25, 2005 at 7. The Examiner later argues that Sakurai et al. teaches that not all inducers of *c-fos* in pericyte cells would be expected to induce angiogenesis. More specifically, the Examiner asserts that Sakurai et al. "describes that the expression of *c-fos* mRNA was induced by FCS (fetal calf serum) and various prostaglandins (see Figure 5); however, only PGD₂ affected the expression levels of VEGF mRNA." *Id.* (emphasis added). The Examiner asserts that Sakurai shows that the skilled artisan would thus "readily appreciate that disclosure that PRO444 polypeptides are capable of stimulation of *c-fos* does not provide any meaningful or definitive evidence that PRO444 molecules could be used as therapeutics in treatment of pathological angiogenesis or any other clinical conditions." *Id.* As discussed below, this is an entirely misleading interpretation of Sakurai et al.

Sakurai et al. is a study that examined the role of prostaglandins in proliferative retinopathy, in which "the underlying mechanism. . . is the formation of new vessels." Sakurai et al. at 2774. The authors hypothesized that prostaglandins, which are well-known inflammatory mediators, may play a role in the development of new vessels. *Id.* To test this hypothesis, the authors examined whether PDG₂ and other prostaglandins for their ability to stimulate proliferation of pericyte cells. As positive and negative controls of pericyte proliferation, the authors cultured pericyte cells in media with or without 10% FCS, respectively. *Id.* at 2775. The authors found that treating pericytes with PDG₂, as well as PGE₂, PGF_{2α} and FCS (the positive control) induced pericyte proliferation. *Id.* at 2776. The authors next assayed the induction of *c-fos* in pericytes treated with prostaglandins or FCS (positive control) and found that when pericytes were treated with PDG₂, PGE₂, PGF_{2α}, and FCS (positive control), the expression of *c-fos* increased. Moreover, the level of pericyte proliferation correlated with the level of *c-fos*

induction. *Id.* at 2777. Because PDG₂ induced pericyte proliferation and *c-fos* expression to the greatest extent, the authors chose PDG₂ as the model to examine whether prostaglandins also induced VEGF expression. *Id.* at 2777-2778. Pericytes treated with PDG₂ showed increased levels of VEGF expression; “a key growth factor in neovascularization.” *Id.* PGD₂ was the only compound that was tested for its effect on VEGF mRNA expression in pericytes.

Sakurai et al. also teaches that PGD₂ mediates induction of *c-fos* and VEGF through the same pathway. *See*, Sakurai et al. at Figures 3 and 8. Specifically, Sakurai et al. reported that PGD₂-mediated induction of *c-fos* is mediated by cAMP. Not surprisingly, a compound that blocks cAMP blocked both PGD₂-mediated induction of *c-fos* and PDG₂-mediated induction of VEGF mRNA. This data confirms the teachings of Janknecht et al. and Kolch et al. described above that state that *c-fos* and VEGF are regulated by the same signal transduction pathways. *See*, Kolch et al. at 141-144; Janknecht et al. at 444-446. Sakurai et al. concluded that their findings provide an explanation “for the known link between angiogenesis and chronic inflammation.” *Id.* at 2780. Notably, the authors referenced Otani et al. (discussed below), and stated that the findings of Sakurai et al. together with the findings of Otani et al. “**further support the view that [] induction of *c-fos* mRNA is an important step in the induction of VEGF expression in retinal pericytes.**” *Id.*

The Examiner’s assertions regarding the Sakurai et al. reference are clearly false. The Examiner argues that Sakurai et al. teaches that only a subset of the factors that induce *c-fos* in turn induce expression of VEGF, but fails to mention that the authors did not even examine whether *c-fos* induction by FCS, PGE₂, or PGF_{2α} stimulated VEGF. Therefore, Sakurai et al. provides no evidence that would lead the skilled artisan to believe that PGE₂, PGF_{2α}, and FCS, as stimulators of *c-fos* expression, would not also induce levels of VEGF expression. To the contrary, the one compound tested for both its ability to induce *c-fos* and to induce VEGF showed that induction of *c-fos* correlated with induction of VEGF. Thus, the teachings of Sakurai et al. would lead the skilled artisan to believe that the compounds that were not tested (*i.e.*, PGE₂, PGF_{2α} and FCS) also induce VEGF expression. That this is indeed the case is demonstrated by the teachings of Kolch et al. that “[b]one formation requires angiogenesis and is strongly stimulated by prostaglandins E1 and E2 (PGE). PGE treatment of osteoblasts increases the expression of VEGF mRNA and protein . . . VEGF induction is blocked by cAMP antagonists.” Kolch et al. at 141. Thus, at least one of the compounds discussed in Sakurai et al.

Appl. No. : 10/066,273
Filed : February 1, 2002

falling within the “subset” of compounds that allegedly induce *c-fos* expression but not VEGF expression, is in fact a known inducer of VEGF.

Accordingly, Sakurai et al. does not support the Examiner’s position. To the contrary, Sakurai et al. is strong evidence in support of Appellants’ asserted utilities.

Otani et al. is the only other reference cited by the Examiner in support of her position that induction of *c-fos* in pericytes cannot support a specific (and presumably a substantial) utility. Like Sakurai et al., Otani et al. concerns *c-fos* expression in pericyte cells. The Examiner argues that Otani et al. teach that both angiotensin II (AII) and VEGF activate *c-fos* in pericytes. *Office Action* mailed March 16, 2005 at 5, demonstrating that “no specific biological function [can] be particularly attributed to PRO444 with respect to its ability to activate *c-fos* expression in pericytes.” *Id.* However, Otani et al. not only fails to provide any support for the Examiner’s assertions, but also confirms that induction of *c-fos* in pericyte cells is recognized as useful by those skilled in the art.

Otani et al. is the continuation of a study that analyzed the role of angiotensin II in retinopathy, which, as discussed in Sakurai et al. above, is attributed to pathogenic neovascularization. Retinal microvasculature is comprised of retinal endothelial cells (ECs) and pericyte cells. Otani et al. at 1192. In a previous study, the authors examined the effect of angiotensin II on retinal endothelial cells. In that report, angiotensin II was shown to potentiate VEGF-mediated angiogenic activity of retinal endothelial cells. That study showed that the angiogenic activity was due to angiotensin II-mediated induction of VEGF receptor expression in retinal epithelial cells. *Id.* In that same study, the authors demonstrated that angiotensin II did not stimulate VEGF expression in retinal endothelial cells.

The experiments disclosed in Otani et al. concern whether and how angiotensin II affects VEGF expression in pericyte cells, the other component of retinal vasculature. The authors found that angiotensin II induces a significant increase in VEGF mRNA in pericyte cells in a time and dose-dependent manner. *See*, Otani et al. Figure 1. The increase in VEGF expression was blocked with antisense *c-fos* oligonucleotides, demonstrating that the induction of VEGF was mediated by *c-fos* expression. Thus, Otani et al. is completely contradictory to the Examiner’s assertion that “there appears to be no evidence of record to show that induction of *c-fos* in pericytes is directly and specifically associated with expression of VEGF.” *Final Office Action* at 5. The authors also demonstrated that the culture media from the angiotensin II treated

pericytes was capable of stimulating the proliferation of retinal endothelial cells, presumably due to the secretion of VEGF by the treated pericytes. The authors conclude that “[t]hese findings suggest that AII might induce angiogenic activity through a paracrine function of VEGF in retinal microvascular cells.” *Id.* at 1192. Thus, the authors expressly state the link between *c-fos* induction in pericytes and stimulation of angiogenesis. The results of these experiments led the authors to suggest that inhibitors of angiotensin might effectively prevent diabetic retinopathy. In other words, due to the specific biological activity of angiotensin II involving induction of *c-fos* in pericyte cells, the authors suggest that antagonists of angiotensin II would be useful for treating pathogenic neovascularization. As such, Otani et al. provides strong evidence that the skilled artisan would believe that PRO444 polypeptides are useful for identifying antagonists to treat pathogenic vascularization, such as pericyte-associated tumors.

c. ***The evidence cited by the Examiner does not establish that pericytes have no known, specific role in angiogenesis***

Appellants turn next to the Examiner’s argument that “there appears to be no information available at the time of filing regarding [the] specific role [of pericytes] in angiogenesis (see Applicant’s cited art). Moreover. . . the post-filing publication of Ozerdem et al, 2003, clearly indicates that it is presently not fully understood if stimulation of pericytes results in up-regulation or down-regulation of vascularization. . . [and that] the art at the time of invention does no [*sic*] substantiate the nexus between stimulation of *c-fos* in pericytes and their involvement, positive or negative, in angiogenesis.” *Final Office Action* at 4-5.

In the Examiner’s own words, “pericytes are reasonably expected to play a significant role in formation of new blood vessels or angiogenesis.” *Final Office Action* at 4. Nevertheless, the Examiner argues that Diaz-Florez et al. and Ozerdem et al. demonstrate that angiogenesis and neovascularization are “very complex” and that the “involvement of pericytes in angiogenesis is controversial and not fully understood.” *Office Action* mailed July 21, 2005 at 3. The Examiner maintains that in view of the above, *c-fos* induction in pericytes “cannot be specifically associated with onset of cancer or angiogenesis as asserted in the Gerritsen Declaration.” *Office Action* mailed March 16, 2005 at 5-6.

Diaz-Florez unambiguously demonstrates that at the time of filing, pericytes had known, specific roles in angiogenesis.

The abstract of Diaz-Florez lists the following “events” in neovascularization:

a) endothelial cell (EC) *and pericyte activation*; b) basal lamina degradation; c) *migration and proliferation of EC and pericytes*; d) formation of a new capillary vessel lumen; e) *appearance of pericytes around the new capillaries*; f) development of a new basal lamina; g) capillary loop formation; h) persistence or involution, and differentiation of the new vessels; and i) capillary network formation and, eventually, organization into larger microvessels. (emphasis added)

Although Diaz-Florez et al. states that angiogenesis is “complex,” and that “*stepwise*, the current model of angiogenesis is controversial,” Diaz-Florez et al. makes it abundantly clear that at the time the instant application was filed, pericytes were known to be involved in several specific steps of angiogenesis. The passage of Diaz-Florez et al. previously cited by the Examiner as demonstrating that the role of pericytes was “controversial,” states that “*most of the authors* are of the opinion that the involvement of capillaries with pericytes occurs at the end of the proliferative stage,” (*Id.* at 818), which Appellants submit is addressed in studies demonstrating the role of pericytes and VEGF in survival of newly formed vasculature, discussed further below. The “controversy” discussed in Diaz-Florez et al. that the Examiner argues weighs against Appellants’ asserted utilities refers to a discussion of studies that had demonstrated that in addition to their role in the survival of newly formed vasculature, studies had shown that pericytes played an early role angiogenesis. More specifically, the referenced studies showed “fusion of pericytes with the endothelium at the point of active angiogenesis. . .and the presence of cytoplasmic processes of pericytes and EC caving in on each other. . .in the early stages of neovascularization. . .[and] nascent pericytes showing cellular processes advancing at the tips of endothelial sprouts. . .suggesting that pericytes may serve as guiding structures of EC outgrowth.” *Id.* Appellants submit that Diaz-Florez et al. does not provide evidence that would lead the skilled artisan to believe that pericytes have no known, specific, roles in angiogenesis and that weigh against Appellants’ asserted utilities. To the contrary, Diaz-Florez et al. enumerates several specific functions of pericyte cells in angiogenesis such as sprout formation/EC proliferation, and survival of newly formed vasculature. Notably, the Examiner does not address Appellants’ assertions regarding the teachings of Diaz-Florez et al. in the Final Office Action or the Advisory Action.

The Examiner next asserts that Ozerdem et al. clearly indicates that it is “presently not fully understood if stimulation of pericytes results in up-regulation or down-regulation of vascularization.” *Final Office Action* at 4. This is an entirely misleading interpretation of Ozerdem et al.

Ozerdem et al. studied the composition of angiogenic sprouts by immunofluorescence. The authors reported the occurrence of pericyte tubes in early carcinoma tumors, and noted the presence of “entire vessels [that] appear to be composed of pericyte tubes,” and “large numbers of individual pericytes invading the tumors.” Ozerdem et al. at 243. Ozerdem et al. also found “the pericytes and endothelial cells are both present at the growing tip of the vascular sprout.” *Id.* This reconfirms the same specific activity of pericytes reported in Diaz-Florez et al. Importantly, the authors concluded that “activated. . . pericytes play an early role in the development of angiogenic sprouts and vessels” and emphasizes “the early participation of pericytes in both physiological and pathological angiogenesis.” *Id.* Ozerdem et al. teaches that “pericytes represent an additional target for treatments designed either to up-regulate (for example in ischemic disorders), or down-regulate (for example in cancer) vascularization.” *Id.* at 248. In other words, rather than providing evidence that establishes that the skilled artisan would doubt Appellants’ asserted utilities, Ozerdem et al. demonstrates *exactly the opposite*, namely that skilled artisans believe that due to their established role in angiogenesis, pericytes are useful in the exact same capacity that Appellants assert in their specification, i.e., as therapeutic targets for treatments where angiogenesis is desirable (ischemia) or where blocking angiogenesis is desirable (cancer). Thus, Ozerdem et al. does not provide evidence to support the Examiner’s *prima facie* showing, but provides strong evidence that that the skilled artisan would believe Appellants’ asserted utilities.

d. The evidence cited by the Examiner does cast doubt on the relationship between c-fos and VEGF

Appellants turn next to the Examiner’s only remaining argument in support of her *prima facie* case of lack of utility: that the relationship between *c-fos* induction and VEGF expression is not “obvious.” The Examiner relies on Orlandini et al. for the proposition that “there is no indication that induction of expression of *c-fos* protooncogene that is known to be induced by many cellular stimuli, including growth factors, cytokines, T-cell activators, UV irradiation, hypoxia and PMA. . . leads to stimulation of VEGF expression by means of AP-1 transcription factor.” *Final Office Action* at 5.

Orlandini et al. describes an *in vitro* differential mRNA screening study conducted on fibroblast cells that differ in the expression of *c-fos*. Inasmuch as Orlandini et al. is completely silent regarding gene expression levels in pericyte cells, it is irrelevant to Appellants asserted

utilities. Orlandini et al. showed that after addition of 10% FCS to culture media, VEGF expression was induced in both *c-fos*^{-/-} fibroblast cells and *c-fos*^{-/-} fibroblast cells engineered to constitutively express exogenous *c-fos*. In view of the data, the authors merely suggest that *c-fos* may not be necessary for VEGF expression in fibroblasts cultured *in vitro*.

Importantly, in the discussion of the results, the authors refer to a study by Saez et al. that “contrasts with [their] results.” Orlandini et al. at 11680. Saez et al. is an *in vivo* study of tumorigenesis in *c-fos*^{-/-} transgenic mice. Saez et al. (1995) *Cell* 87:721-732. Insofar as Saez et al. is not limited to fibroblast cells, Appellants assert that it is more probative of Appellants’ asserted utilities than Orlandini et al. Saez et al. teaches that tumors in transgenic *c-fos*^{-/-} mice “show[] very little external vascularization.” Saez et al. at 723. This finding prompted the authors of Saez et al. to examine the mRNA levels of “certain AP-1 regulated genes” in tumors from *c-fos*^{-/-} and *c-fos*^{+/+} mice, including VEGF. The authors found that VEGF mRNA levels were 5-10 fold lower in the tumors from the *c-fos*^{-/-} mice compared to tumors from *c-fos*^{+/+} mice, thereby demonstrating that *c-fos* is necessary for the full induction of VEGF and vascularization of tumor cells.

As shown above, Orlandini et al. is insufficient to support a *prima facie* case of lack of utility. First, Orlandini et al. concerns a different cell type than the specialized pericyte cells used in Example 60 of the instant application. Accordingly, Orlandini et al. is irrelevant to Appellants’ asserted utilities and provides no evidentiary weight that the skilled artisan would doubt Appellants’ utilities. Even if Orlandini et al. was relevant to Appellants’ asserted utilities, the authors of Orlandini et al. question their own conclusions about *c-fos* regulation of VEGF in view of Saez et al., which is more probative of Appellants’ asserted utilities. Saez et al. demonstrates the link between *c-fos* induction, VEGF induction, and vascularization of tumors. Thus, the totality of the evidence weighs in favor of Appellants’ asserted utilities.

e. **Conclusion – The Examiner has failed to establish a prima facie case that one of skill in the art would doubt Appellants’ asserted utility**

The Examiner has relied on essentially three arguments in rejecting the pending claims for lack of utility. First, the Examiner presents several articles demonstrating that various stimuli induce *c-fos* in various cell types, and argues that in view of these references, the ability to induce *c-fos* in pericytes “does not provide any meaningful or definitive evidence that PRO444 could be used as therapeutics in treatment of pathological angiogenesis or any other clinical

conditions.” *Final Office Action* at 7. Second, the Examiner proffers two articles that allegedly demonstrate that the role of pericytes in angiogenesis is not well understood, and argues that the references show that the skilled artisan would not believe that induction of *c-fos* in pericytes is associated with cancer or the onset of angiogenesis. Finally, the Examiner argues that the connection between *c-fos*, AP-1, and VEGF is “not obvious” relying on Orlandini et al. as support. Appellants have responded to each of these arguments in turn.

Appellants have shown that the data in Example 60 are sufficient to establish that PRO444 is useful in the treatment of certain tumors and as a stimulator of angiogenesis. The Examiner has not provided any relevant reason or evidence for one of skill in the art to doubt the usefulness of PRO444.

Next Appellants have shown that each and every reference relied upon by the Examiner is either irrelevant to Appellants’ asserted utilities or provides further evidence of Appellants’ asserted utilities. First, Appellants have shown that Janknecht et al., Herrera et al., Kovács et al., and Coulon et al. fail to mention pericyte cells or *c-fos* activation in pericyte cells. Thus, they carry no evidentiary weight in establishing a *prima facie* case that the skilled artisan would question the truth of Appellants’ asserted utilities. Moreover, Janknecht et al. establishes that *c-fos* and VEGF are both regulated by the same signals (e.g., through the Ras/Raf-1 and cAMP pathways), and confirms that it was known in the art that *c-fos* is a transcriptional regulator involved in cellular proliferation and differentiation. Herrera et al. propose that *c-fos* is involved in wound healing in the brain. Appellants have shown that the two references relied upon by the Examiner that particularly concern *c-fos* induction in pericytes, Sakurai et al. and Otani et al., teach that *c-fos* induction leads to VEGF expression in pericytes and that antagonists of inducers of *c-fos* in pericytes are useful in treating pathogenic neovascularization, respectively, thereby providing strong evidence in support of Appellants’ utilities. Second, Appellants have shown that Diaz-Florez et al. and Ozerdem et al. both teach that pericytes have known functions in both early and late stages of angiogenesis by promoting cellular proliferation and by facilitating the survival of newly formed vasculature. Finally, Appellants have shown that Orlandini et al. is not probative of Appellants’ asserted utilities since it concerns *c-fos* expression in fibroblast cells and not pericyte cells and that Orlandini et al. explicitly states that *in vivo* studies of Saez et al. directly contradict the conclusions regarding *c-fos* induction of VEGF expression that the authors draw from their own data. Thus, Orlandini et al. is not strong evidence regarding regulation of

VEGF expression overall, and provides no evidence regarding VEGF expression in pericyte cells. Notably, Saez et al., which is not limited to fibroblast cells, teaches that *c-fos* correlates with increased VEGF mRNA levels in tumors, and that tumors in *c-fos*-deficient mice lack external vascularization. In other words, Saez et al. provides a link between *c-fos* expression, VEGF expression, and vascularization, fully consistent with Appellants' asserted utilities.

Taken together, the Examiner's arguments are not sufficient to satisfy the Examiner's burden to "provide[] evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The Examiner's arguments are not supported by any substantial relevant evidence or reasoning which explains why one of ordinary skill in the art would reasonably doubt the asserted utility. Therefore, the Board should accept the Appellants' disclosure of utility. *See Ex parte Rubin*, 5 U.S.P.Q. 2d 1461 (Bd. Pat. App. & Interf. 1987) ("There is no factual support in this record for the examiner's questioning of the denaturation test reported in the specification. ... No reason to doubt 'the objective truth' of the asserted utility having been advanced by the examiner, we accept appellant's disclosure of utility corresponding in scope to the claimed subject matter.").

7. Appellants have provided Sufficient Rebuttal Evidence of Utility

"Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The rebuttal evidence must be sufficient such that when it is considered as a whole, it is more likely than not that the asserted utility is true. *See In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992) (stating that the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or "more likely than not" standard). The M.P.E.P. summarizes the standard of proof required:

[T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. *M.P.E.P.* § 2107.02, part VII (emphasis in original, citations omitted).

Appl. No. : 10/066,273
Filed : February 1, 2002

Appellants remind the Board that the Federal Circuit has stated that the standard for satisfying the utility requirement is a low one: "The threshold of utility is not high: An invention is 'useful' under section 101 if it is capable of providing some identifiable benefit." *Juicy Whip, Inc. v. Orange Bang, Inc.*, 185 F.3d 1364, 1366, 51 U.S.P.Q. 2d 1700 (Fed. Cir. 1999).

Even if the Examiner has satisfied her burden of presenting a *prima facie* case of lack of utility, Appellants have supplied more than enough rebuttal evidence, such that when considered as a whole, one of skill in the art would conclude that the asserted utility is more likely than not true. Specifically, Appellants have provided evidence that at the time of filing of the instant application, pericytes were known to have specific roles in angiogenesis in both the formation of new blood vessels and the survival of newly formed vasculature. In addition, Appellants have provided evidence demonstrating that at the time of filing of the instant application, VEGF was known to be a potent angiogenic factor involved in both cellular proliferation in connection with neovascularization, as well as survival of newly formed vasculature. Appellants also provided evidence that at the time of filing *c-fos* was well known to function as a component of the AP-1 transcription factor, which was a recognized regulator of VEGF expression. Finally, in addition to the evidence relied upon by the Examiner in which skilled artisans have articulated the very same utilities for regulators of *c-fos* in pericytes as set forth in Appellants' specification, Appellants have provided evidence that skilled artisans have in fact looked to upstream regulators of VEGF expression similar to PRO444 as targets for blocking angiogenesis in tumor therapy. Therefore, considering the evidence as a whole, one of skill in the art would not doubt that the claimed antibodies are useful as therapeutic targets for pericyte-associated tumors and as stimulators of angiogenesis.

a. Appellants have established that pericytes have established specific roles in angiogenesis

As discussed above, the Examiner has not provided any relevant evidence or reasoning that demonstrates that pericytes do not have specific roles in angiogenesis recognized by those skilled in the art. In contrast to this complete lack of relevant evidence on the part of the Examiner, Appellants have submitted several references that pre-date the instant application that discuss specific roles of pericytes in angiogenesis.

Appl. No. : 10/066,273
Filed : February 1, 2002

Nehls et al. ((1992) *Cell Tissue Res.* 270:469-474) describes pericyte involvement in capillary sprouting during angiogenesis *in situ*. The authors induced angiogenesis in mouse mesentery tissue and used immunofluorescence to identify pericytes. The authors found that pericytes were regularly positioned at and in front of the advancing tips of endothelial sprouts, and bridging gaps between the leading edges of endothelial sprouts. The authors concluded that pericytes are involved in capillary sprouting.

Rhodin et al. also found that pericytes are regularly found in association with capillary sprouts. Rhodin et al., (1989), *J. Submicrosc. Cytol. Pathol.* 21:1-34, 12. As such, the results of Rhodin reinforce the conclusions reached by Nehls, *i.e.*, that pericytes are involved in capillary sprouting.

Diaz-Florez et al. is a review article published prior to the filing date of the present application and was originally cited by the Examiner in the Office Action mailed July 21, 2005. Diaz-Florez demonstrates that at the time of filing, pericytes had known, specific roles in angiogenesis. More specifically, Diaz-Florez states that “*most of the authors* are of the opinion that the involvement of capillaries with pericytes occurs at the end of the proliferative stage,” (*Id.* at 818), which Appellants submit is addressed in studies demonstrating the role of pericytes in survival of newly formed vasculature, discussed further below. Diaz-Florez also addresses studies that show that pericytes play an early role angiogenesis. Other studies referred to in Diaz-Florez showed “fusion of pericytes with the endothelium at the point of active angiogenesis. . .and the presence of cytoplasmic processes of pericytes and EC caving in on each other. . .in the early stages of neovascularization. . .[and] nascent pericytes showing cellular processes advancing at the tips of endothelial sprouts. . .suggesting that pericytes may serve as guiding structures of EC outgrowth.” *Id.* In the final Office Action, the Examiner does not address Appellants’ assertions regarding the specific teachings of Diaz-Florez.

In addition to these references, Appellants previously submitted review articles by Balabanov et al. and Ellis et al. that summarize what was known at the time the instant application was filed regarding the role of pericytes in angiogenesis. Balabanov et al. was published four months after the filing date of the instant application, and echoes the teachings of Nehls et al., Rhodin et al. and Diaz-Florez et al., above, stating:

[p]ericytes have been implicated in all three stages of new vessel formation: 1) initiation, 2) sprout extension and migration, 3) maturation and cessation of growth. (Hirshi and D’Amore, 1997) At the initiation phase pericytes respond to

Appl. No. : 10/066,273
Filed : February 1, 2002

a number of angiogenic stimuli. As a result, they undergo activation, degrade the basement membrane, and migrate out of the microvessels. (Diaz-Florez et al., 1994). Pericytes guide the migrating endothelial cell, regulate their proliferation, and form connections between newly formed sprouts (Hirshi and D'Amore, 1997; Diaz-Florez et al., 1994; Nehls et al., 1992). Such functions are thought to be mediated through TGF β -1 (Orlidge and D'Amore, 1987; Sato and Rifkin, 1989), vascular endothelial growth factor (Hirshi and D'Amore, 1997, Kim et al., 1998). Balabanov et al. at 640.

Each and every reference cited within Balabanov et al. in the passage above was published prior to Appellants' filing date.

Ellis et al. is yet another review article concerning the role of pericytes in angiogenesis as it relates to tumor biology. Ellis, (2002), *Oncology* 16(5):14-22. Ellis explains that "the tumor microenvironment is a caustic one. . . [t]herefore, for these fragile endothelial cells [that represent the new primitive capillary network] to survive, they must be exposed to endothelial cell survival factors. . . Endothelial cell survival factors include pericytes that may stabilize endothelium. . . by secretion of endothelial cell survival factors such as VEGF." Ellis, at 20. As such, Ellis et al. underscores the fact that it was known that one of the roles in angiogenesis that pericytes play is to promote survival of newly formed vasculature by secreting VEGF.

Finally, Ozerdem et al., entitled "Early contribution of pericytes to angiogenic sprouting and tube formation," is a publication that was originally cited by the Examiner in the Office Action dated March 16, 2005 for the proposition that pericytes "role in formation of tumor neovasculature is currently not fully understood and varies depending on type of tissue and tumor (see page 241, 242, and 246)." To further refine studies regarding the role of pericytes in vascularization of tumors, Ozerdem et al. utilized immunofluorescence to analyze the composition of angiogenic sprouts. The authors found the occurrence of pericyte tubes in early carcinoma tumors, noting the presence of "entire vessels [that] appear to be composed of pericyte tubes," and "large numbers of individual pericytes invading the tumors." Ozerdem et al. at 243. Ozerdem *et al.* also found "the pericytes and endothelial cells are both present at the growing tip of the vascular sprout." *Id.* The authors conclude that "activated. . . pericytes play an early role in the development of angiogenic sprouts and vessels." and emphasize the early participation of pericytes in both physiological and pathological angiogenesis. *Id.* These findings and conclusions reinforce the findings and conclusions in the earlier studies of Nehls, Rhodin and the studies discussed in Diaz-Florez et al. and Balabanov et al. Even further,

Ozerdem et al. provides the suggestion that, due to their specific roles in angiogenesis, “pericytes represent an additional target for treatments designed either to up-regulate (for example in ischemic disorders), or down-regulate (for example in cancer) vascularization.” *Id.* at 248. This unambiguously demonstrates that the skilled artisan would believe Appellants’ asserted utilities.

Finally, Appellants previously submitted a Declaration by Dr. Mary Gerritsen as evidence in support of the asserted utilities. Dr. Gerritsen testifies that “pericytes help regulate capillary permeability and stabilize newly formed blood vessels” and that “pericytes play an important role in regulating angiogenesis.” (Gerritsen Decl., ¶6). The pre-filing publications of Nehls et al., Rhodin et al. and Diaz-Florez et al., the pre-filing publications referenced in Balabanov et al., as well as Ellis et al. and Ozerdem et al. all illustrate the same, specific roles for pericytes in angiogenesis testified to by Dr. Gerritsen. The Examiner has offered no reasoning or evidence that contradicts Appellants’ evidence, including the teachings of the references discussed above and Dr. Gerritsen’s testimony, or establishes that the skilled artisan would have a legitimate basis to doubt the credibility of Dr. Gerritsen’s testimony.

The Examiner’s assertion that “there appears to be no information available at the time of filing of [pericyte’s] specific role in angiogenesis,” (*Final Office Action* at 4) is incorrect in view of the evidence discussed above. In fact, all of the evidence of record concerning pericytes demonstrates that at the time the instant application was filed, pericytes were known to be involved in angiogenesis in at least two capacities: endothelial sprout formation and survival of newly formed vasculature.

b. Appellants have established that VEGF has an established role in angiogenesis

Appellants next turn to the second portion of their argument in support of their asserted utility – that VEGF has well-established roles in angiogenesis, both in inducing cellular proliferation and vascular permeability, and in promoting survival of newly-formed vasculature. In the section above, Appellants provided several pre-filing references that demonstrated that these same activities are mediated by pericyte cells.

At the time of filing of the instant application, studies had demonstrated that VEGF is involved in survival of endothelial cells in newly formed vessels. Alon et al., (1995), *Nat. Med.* 1(10):1024-1028, examined the role of VEGF in retinopathy of prematurity (ROP), a disorder

that ultimately results in blindness. It was generally accepted at the time that VEGF caused the abnormal vasoproliferation in ROP. Alon et al. showed that the absence of VEGF during the early stage in ROP resulted in blood vessel regression. Exogenously added VEGF reversed this process. Thus, Alon et al. concluded that VEGF is involved in survival of newly formed vasculature.

The studies of Benjamin et al. (1997), *Proc. Nat. Acad. USA* 94:8761-8766, confirm the role of VEGF in survival of newly-formed vasculature reported in Alon et al. Benjamin et al. engineered tumor cells in which VEGF expression could be induced or shut off. These cells were then injected into mice to study the effects of VEGF expression on tumor vascularization. Not surprisingly, the authors found that over expression of VEGF results in tumor hypervascularity. On the other hand, when VEGF expression was shut off, preformed tumor vessels regressed. Benjamin et al. concluded "VEGF is required for maintenance of . . . tumor vessels." *Id.* at 8762. In a section entitled "Clinical Implications of Vascular Regression Caused by VEGF Withdrawal," Benjamin et al. state that the "finding that newly formed or remodeling blood vessels require sustained VEGF levels will be critical in the success of many angiogenic and anti-angiogenic therapies." *Id.* at 8675.

Appellants also submitted Ferrera et al. ((1995) *Breast Cancer Res. and Treat.* 36:127-137) as evidence demonstrating the involvement of VEGF in inducing cellular proliferation involved in angiogenesis. Ferrera et al. cite several references showing that "VEGF is a potent mitogen. . .for vascular endothelial cells." *Id.* at 128. Ferrera et al. also reference early studies demonstrating that VEGF promotes angiogenesis in a tridimensional *in vitro* model, inducing confluent microvascular endothelial cells to invade a collagen gel and form tube-like structures. *Id.* In addition, Ferrera et al. teaches that it was known that VEGF promotes vascular permeability for vascular endothelial cells. Ferrera, et al. at 127. Ferrera et al. teaches that prior to 1995, VEGF was thought to function in a paracrine fashion. Ferrera et al., at 128. Notably, the study of Otani et al., submitted by the Examiner, confirms the earlier teachings of Ferrera et al., stating that "VEGF. . .produced by pericytes, induce[s] endothelial cell growth in a paracrine manner indicat[ing] a proliferative effect of pericytes." Otani et al., at 1197.

Appellants submit that the references cited above demonstrate that at the time the Application was filed, those skilled in the art appreciated the critical role of VEGF in angiogenesis, as required for inducing proliferation of vascular endothelial cells, survival of

newly formed vasculature, and vascular permeability. This led Kolch et al. to describe VEGF as “the pivotal mediator of pathophysiological angiogenesis.” Kolch et al. at 139. The Examiner has not offered any evidence that calls into question Applicants’ assertions.

The Examiner has conceded that “the role of angiogenic [*sic*] factor VEGF is well established.” *Final Office Action* at 5. The Examiner has also conceded that “[t]here is also no dispute that the art at the time of filing discloses that pericytes could secrete VEGF.” *Id.* Nevertheless, the Examiner maintains that “the art at the time of the invention does no [*sic*] substantiate the nexus between stimulation of *c-fos* in pericytes and their involvement, positive or negative, in angiogenesis.” *Office Action* at 5. Appellants submit that the totality of the evidence clearly demonstrates the relationship between VEGF expression and the role of pericyte involvement in angiogenesis. As discussed below, Appellants have also provided several references demonstrating that *c-fos* is a known regulator of VEGF expression.

c. Appellants have established that *c-fos* stimulates VEGF expression

Appellants next turn to the final portion of their argument in support of their asserted utility - that at the time of filing, *c-fos* was known to be part of transcription factor AP-1, and that AP-1 was known to regulate VEGF expression.

The evidence submitted by the Examiner demonstrates that at the time of filing, it was well known that *c-fos* is a component of the AP-1 transcription factor. Citing to a textbook on transcriptional regulators, Janknecht teaches that “the *c-fos* gene encodes a basic region-leucine zipper transcription factor that requires heterodimerization with a member of the Jun family for stable DNA binding. Fos/Jun heterodimers are present in the AP-1 transcription factor.” Janknecht at 443. Thus, there is a clear, “obvious” relationship between *c-fos* and AP-1.

Appellants previously submitted Tischer et al. (1991), *J. Biol. Chem.* 266(18):11947-11954; Shima, et al., (1996) *J. Biol. Chem.* 271(7):3877-3882, 3882; and Kolch et al. as evidence that at the time of filing, AP-1 was known to regulate VEGF expression.

In 1990, Tischer et al. analyzed the human gene for VEGF. Tischer et al. (1991), *J. Biol. Chem.* 266(18):11947-11954. The authors found that the promoter region for hVEGF contains several AP-1 binding sites, suggesting that *c-fos* is a regulator of VEGF expression. Tischer at 11953. Similarly, the structure of the mouse VEGF gene revealed “multiple consensus binding sties for AP-1.” Shima, et al., (1996) *J. Biol. Chem.* 271(7):3877-3882, 3882. In Kolch’s review “Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis,”

Appl. No. : 10/066,273
Filed : February 1, 2002

Kolch summarizes the state of the art in 1995 stating that “[a]t present, a comprehensive assessment of several studies highlights the AP-1 transcription factor as an important common denominator for the regulation of VEGF expression.” Kolch, at 144, emphasis added.

The art available at the time of filing demonstrates that *c-fos* and VEGF are regulated through the same signal transduction pathways, explaining the fact that regulators that function to stimulate *c-fos* also stimulate VEGF expression. More particularly, Kolch et al. highlights various pathways in which both *c-fos* and VEGF expression are regulated, including through the Ras/Raf pathway. *Id.* at 144-145. Janknecht et al. teaches that the Ras/Raf-1 pathway is involved in regulation of *c-fos* by both growth factors and UV light. Janknecht et al. at 444-445. Importantly, Kolch et al. also links the induction of *c-fos* expression through the Raf and Ras pathways with conversion to a tumorigenic phenotype through activation of VEGF. *Id.* at 145. Janknecht et al. also teaches that *c-fos* is regulated by cAMP. As discussed above, Kolch et al. and Sakurai et al. both teach that VEGF is regulated by cAMP. Kolch et al. at 141; Sakurai et al. at 2780. Thus, in view of the fact that the same pathways regulate both *c-fos* and VEGF, the Examiner’s assertions that since growth factors and UV light induce *c-fos*, the skilled artisan would not believe Appellants’ asserted utilities are not convincing. Rather, in view of Janknecht et al. and Kolch et al., the skilled artisan would be led to believe that more likely than not, the same stimuli would also induce VEGF expression. Thus, the literature at the time of filing establishes the link between *c-fos* regulation and VEGF regulation.

Appellants also previously submitted a review article by McColl et al. which articulates Appellants’ reasoning regarding the specificity of molecules such as PRO444 in the following statement: “since *fos* is upregulated by [various stimuli including growth factors], VEGF expression could also be elevated in response to these stimuli, *as is indeed the case.*” McColl et al., (2004) APMIS 112:463-480, 467 and references cited therein (emphasis added).

Appellants submit that the references discussed above demonstrate that at the time the instant application was filed, those skilled in the art appreciated the role of *c-fos* in VEGF expression, and hence, the role of *c-fos* in the angiogenic process, including neovascularization and stabilization of newly formed vasculature.

d. *Appellants have established that skilled artisans believe that indirect regulators of angiogenic factors are useful as therapeutic targets for cancer therapy*

As Dr. Gerritsen testified, “a skilled artisan would reasonably conclude that neutralizing compounds capable of stimulating *c-fos* expression in pericytes (e.g., PRO444) could be useful in preventing the onset and/or progression of cancer and/or angiogenesis.” Gerritsen Decl., ¶6. The numerous references of record that are discussed above fully support this proposition. As even further proof of this principle, however, Appellants previously submitted a review article entitled “Synopsis of Angiogenesis Inhibitors in Oncology.” Ellis et al., (2002) *Oncology* 16(5):14-22. Ellis et al. teaches that in 2002, scientists had proposed and had been working towards developing anti-angiogenic therapies for the treatment of cancer that fell into four different categories: (1) those that decrease the activity of specific angiogenic factors; (2) those that decrease the activity of endothelial survival factors; (3) those that increase the activity of naturally occurring anti-angiogenic agents; and (4) those that indirectly downregulate activity of angiogenic and survival factors. Ellis et al. at 18 (emphasis added). For the purposes of discussion in the review, Ellis et al. use VEGF as “the prototype molecule used to describe strategies to decrease the activity of angiogenic factors.” *Id.* In the discussion of the second strategy, Ellis et al. states that “[e]ndothelial cell survival factors include pericytes that may stabilize endothelium. . .by secretion of endothelial cell survival factors such as VEGF.” In other words, Ellis et al. teaches that by 2002, skilled artisans had been looking to factors that regulate VEGF expression in pericyte cells as cancer therapeutics. Furthermore, in the discussion of the fourth strategy, Ellis et al. states that “strategies that downregulate the upstream signaling pathways to VEGF. . .may indirectly downregulate VEGF activity and angiogenesis.” *Id.* at 20. At the time of filing, it was known that *c-fos* represented an upstream signaling pathway to VEGF. *See*, Kolch et al. 145. As such, Ellis et al. teaches that by 2002, skilled artisans had been looking at factors to downregulate molecules such as *c-fos* as cancer therapeutics. Ellis et al. provides direct evidence that demonstrates that skilled artisans had actually contemplated the use of upstream regulators of VEGF (e.g., PRO444) to identify antagonists for use in cancer therapy.

As even further proof of Appellants’ asserted utilities, Appellants also previously submitted evidence demonstrating that the first strategy proposed by Ellis, *i.e.*, directly targeting or neutralizing the activity of angiogenic factors such as VEGF, has been demonstrated

to be effective. A VEGF-specific antibody, bevacizumab, has been successfully used to treat several cancer types. See, Kirkpatrick, P., (2005), *Nat. Rev. Drug Disc.* S8-S9. Willett et al. report that bevacizumab has antivasular effects in human rectal cancer. Willett et al. (2004) *Nature Medicine*, 10(2):145-147. Regardless of their publication date, Ellis et al. Kirkpatrick, and Willett et al. summarize years of study involving the identification of regulators of VEGF as cancer therapeutics, and each is irrefutable proof of the truth of Appellants' asserted utilities.

e. Conclusion

As a whole, the evidence of record establishes that skilled artisans more likely than not believe that pericyte cells function in angiogenesis by inducing proliferation of vascular endothelial cells and promoting survival of newly formed vasculature; that VEGF is a potent angiogenic factor that induces proliferation of vascular endothelial cells and promotes survival of newly formed vasculature; and that *c-fos* is directly involved in VEGF expression, particularly in pericyte cells. Given the overwhelming amount of evidence in support of Appellants' position, including publications that expressly articulate Appellants' asserted utilities, and the near absence of any relevant evidence in support of the Examiner's position, when considered as a whole, the evidence leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true.

8. *The Examiner's Response to Appellants' Evidence is Insufficient to Rebut Appellants' Arguments*

The Examiner has concluded that the teachings of the specification, the scientific references, the declaration of Dr. Gerritsen, and supporting arguments provided by Appellants are not persuasive. See, e.g., *Final Office Action* at 8.

a. The Examiner's Response to the Nehls, Rhodin, Diaz-Florez, Balabanov and Ellis references

Appellants submitted several references including Nehls et al., Rhodin et al., Diaz-Florez et al. Balabanov et al., and Ellis et al. as evidence that prior to the filing date of the instant application, those skilled in the art appreciated the specific roles of pericytes in angiogenesis as both stimulators of endothelial cell proliferation and as mediators of survival of newly formed vasculature. In response to Appellants' evidentiary showing, the Examiner states:

Beginning at page 7 of the Response, Applicant submits that at the time of the filing, the role of pericytes in angiogenesis was fully established and refers to

Appl. No. : 10/066,273
Filed : February 1, 2002

articles by Nehls et al., Phodin et al. [*sic*] and Ozerdam et al. [*sic*] (the last cited by the Examiner in the previous office action of record). First, it is important to clarify that the Examiner never disputed that pericytes have a role in angiogenesis. Anatomically, as part of vasculature, pericytes are reasonably expected to play a significant role in formation of new blood vessels or angiogenesis. However, *there appears to be no information available at the time of filing regarding their specific role in angiogenesis* (see Applicant's cited art). Moreover, information presented in post-filing publication of Ozerdem et al., 2003, clearly indicates that it is presently not fully understood if stimulation of pericytes results in up-regulation or down regulation of vascularization (middle at page 8 of the Response). More importantly, the art at the time of invention does not [*sic*] substantiate the nexus between stimulation of *c-fos* in pericytes and their involvement, positive or negative, in angiogenesis (see specifically Applicant's reasoning on pages 10-11 of the Response). *Final Office Action*, at 4-5 (emphasis added).

As an initial matter, Appellants' Response to Office Action never alleged that the role of pericytes in angiogenesis was "fully established." Rather, Appellants stated that the several references "illustrat[e] the state of the art regarding pericyte control of angiogenesis at the time the application was filed." *Amendment and Response to Office Action* mailed October 18, 2005. As described above, Nehls et al., Rhodin et al., and Diaz-Florez et al. are each references published prior to Appellants' effective filing date that enumerate specific functions of pericyte cells in angiogenesis (e.g., proliferation and survival of newly formed vasculature). Without addressing the contents of these references, the Examiner makes the conclusory statement that "there appears to be no information available. . . regarding [pericyte's] specific role in angiogenesis." *Final Office Action* at 4.

The Examiner is silent regarding Appellants' assertions regarding the teachings of Diaz-Florez et al., originally submitted by the Examiner in support of her assertion that "the art teaches that process [*sic*] angiogenesis or neovascularization is controversial and not fully understood." *Office Action* mailed July 21, 2005 at 3.

The Examiner also fails to specifically address Balabanov et al. As Appellants pointed out in the Amendment and Response filed on October 15, 2005, Balabanov et al. is a review article that summarizes the state of the art at the time the instant application was filed. Balabanov et al. was received for publication in June 1998, a mere four months prior to Appellants' priority date of October 28, 1998. In the *Amendment and Response* mailed October 18, 2005, Appellants cited to a passage in Balabanov et al. that in turn cites to several pre-filing

Appl. No. : 10/066,273
Filed : February 1, 2002

references that enumerate the specific roles of pericytes in angiogenesis and attribute several of the specific angiogenic functions of pericytes to the secretion of VEGF. Although the Examiner fails to mention Balabanov et al., assuming that the Examiner's dismissal of this reference as evidence is based on the publication date of Balabanov et al., Appellants maintain that the Examiner's failure to consider the evidence is without merit.

As discussed above, the Examiner never fairly addressed Appellants' citation to the Ozerdem et al. reference (originally cited by the Examiner) as evidence that supports rather than disproves Appellants' asserted utilities. As discussed above, Ozerdem et al. reconfirms the teachings of Nehls et al. and Rhodin et al. demonstrating that pericytes are present at the tips of angiogenic sprouts, conclude that "activated. . . pericytes play an early role in the development of angiogenic sprouts and vessels," and underscore the early participation of pericytes in both physiological and pathological angiogenesis. In response, the Examiner mischaracterizes Appellants' citation of Ozerdem et al., making the misleading assertion that "information presented in post-filing [*sic*] publication of Ozerdem et al., 2003, clearly indicates that it is presently not fully understood if stimulation of pericytes results in up-regulation or down-regulation of vascularization." *Final Office Action* at 5. Surprisingly, the Examiner maintains this position in the Advisory Action even after Appellants pointed out that the Examiner mischaracterized or misinterpreted the reference and that Ozerdem et al. teaches that, like Appellants have asserted, depending on the type of disorder (*e.g.* ischemia or cancer), the skilled artisan would recognize that pericytes are useful targets to either induce or inhibit vascularization.

Finally, the Examiner dismisses the publication of Ellis et al., stating that "with respect to the publications used in discussion on pages 12-13 (including Ellis et al.), Applicant is advised that the asserted utility cannot be relied upon disclosure [*sic*] available after the filing date of the instant specification. As with Balabanov et al., Ellis et al. is a review article that summarizes the state of the art as reflected by several studies published to date, many of which, as Appellants' have pointed out, pre-date the filing date of the instant application.

b. The Examiner's Response to the Alon, Ferrera, Pepper, and Benjamin, References

Appellants submitted the Alon et al., Ferrera et al., Benjamin et al., and Pepper et al. references as evidence that at the time of filing, VEGF was a well-known angiogenic factor that has biological activities such as inducing proliferation of endothelial cells, promoting survival of

Appl. No. : 10/066,273
Filed : February 1, 2002

endothelial cells, and inducing vascular permeability - specific activities that were attributed to pericyte cells at the time of filing of the instant application.

In response to Appellants' evidentiary showing, the Examiner concedes that "the role of VEGF is well established. There is also no dispute that the art at the time of filing discloses that pericytes could secrete VEGF." *Final Office Action* at 5.

c. ***The Examiner's Response to the Tischer, Shima, Kolch and Janknecht References***

Appellants submitted the Tischer et al., Shima et al., Kolch et al. and Janknecht et al. references as evidence that at the time of filing, the skilled artisan appreciated that *c-fos* is a subunit of AP-1, a known regulator of VEGF expression. In response to Appellants' evidentiary showing, the Examiner states:

Applicant argues at pages 11-12 that because *c-fos* encodes a subunit of the nuclear transcription factor AP-1 and because AP-1 plays a role in the expression of VEGF, then *c-fos* stimulates VEGF expression. Applicant's arguments as well as presented articles by Tischer et al., Shima et al., and Kolch have been fully considered but are not persuasive **because the relationship between *c-fos*, AP-1 and VEGF expression is not obvious**. Applicant's reasoning lacks support in the specification as originally filed and also in the publications of record because there appears to be no indication that induction of expression of *c-fos* protooncogene that is known to be induced by many cellular stimuli, including growth factors, cytokines, T-cell activators, UV irradiation, hypoxia and PMA (see reasoning in the previous office actions of record and also Orlandi [*sic*] et al, 1996, Proc. Natl. Acad. Sci., USA, Vol. 93, pp. 1675 [*sic*]-11680) leads to stimulation of VEGF expression by means of AP-1 transcription factor. On the contrary, Orlandi [*sic*] et al. publication discloses that, for example, in fibroblasts VEGF expression is unaffected by *c-fos*. *Final Office Action* at 7 (emphasis added).

As demonstrated above, the Examiner has offered no reasoning or evidence that contradicts Appellants' evidentiary showing of Janknecht et al. that *c-fos* is a subunit of the AP-1 transcription factor. The Examiner has offered no reasoning or evidence that contradicts Appellants' evidentiary showing of Tischer et al. and Shima et al. that the regulatory region of the VEGF gene contains AP-1 binding sites. As such, the skilled artisan would believe that inducers of *c-fos* would in turn induce VEGF expression. Appellants have provided evidence that establishes that *c-fos* and VEGF are in fact regulated by the same signal transduction pathways, *e.g.*, Ras/Raf-1 and cAMP, and that *c-fos* functions to induce VEGF expression. The only evidence offered by the Examiner to contradict Kolch et al., which states that "a

comprehensive assessment of several studies highlights the AP-1 transcription factor as an important common denominator for the regulation of VEGF expression,” is an article by Orlandini et al. As discussed above, Orlandini et al. is not contradictory to Appellants’ evidence. Orlandini et al. is a study done in fibroblast cells, and therefore has no bearing on the regulation of VEGF expression in pericyte cells. Furthermore, Orlandini et al. call into question this very conclusion in view of the evidence that *c-fos* regulates VEGF expression *in vivo*.

d. The Examiner’s Response to the Gerritsen Declaration

Appellants submitted a declaration under 37 C.F.R. § 1.131 by Dr. Mary Gerritsen as evidence that the compounds identified in Example 60, *e.g.*, PRO444, have a specific biological activity, and that induction of *c-fos* in pericytes is not a generalized response. Dr. Gerritsen’s testimony was also provided as evidence that pericytes are unique cells that have specific roles in angiogenesis, including both the stabilization of newly formed blood vessels and the regulation of capillary permeability.

In response to Dr. Gerritsen’s testimony, the Examiner states that “the Declaration of Gerritsen represents Dr. Gerritsen’s own conclusions with no references to scientific publications.” *Office Action* mailed March 16, 2005 at 4. According to the Examiner, the testimony in the Gerritsen declaration further establishes that “there appears to be no specific biological function that could be particularly attributed to PRO444 with respect to its ability to activate *c-fos* expression in pericytes,” and that “there appears to be no clear physiological meaning attributed to the activation of *c-fos* by PRO444 at the time of filing.” *Id.* at 5-6. In the final Office Action, the Examiner maintains that “the Declaration is insufficient to overcome the instant rejection because it does not provide support for the relationship between expression of *c-fos* in pericytes and angiogenesis.” *Final Office Action* at 6.

Appellants maintain that the Examiner has not heeded the admonition that “Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned.” *M.P.E.P.* § 2107. In addition, declarations relating to issues of fact should not be summarily dismissed as “opinions” without an adequate explanation of how the declaration fails to rebut the Examiner’s position. *See in re Alton* 76 F.3d 1168 (Fed. Cir. 1996). As an initial matter, Appellants have submitted 15 references in addition to the several references cited by the Examiner, which confirm the testimony of Dr. Gerritsen and provide evidence of the truthfulness of Appellants’ assertions. These references confirm Dr. Gerritsen’s

testimony that “retinal pericytes are unique cells that play an important role in . . . angiogenesis. . . [by] regulat[ing] capillary permeability and stabiliz[ing] newly formed blood vessels.” Gerritsen Decl. ¶6. *See, e.g.*, Balabanov et al.; Alon et al.; Benjamin et al.; Ferrera et al. The evidence of record, including Janknecht et al., Kolch et al., and McColl et al., also confirms Dr. Gerritsen’s testimony that “C-fos transcription factor is involved in the regulation of cellular growth, including cancer and angiogenesis. Growth factors capable of stimulating pericytes signal through the *c-fos* pathway.” Gerritsen Decl. ¶6. *See, e.g.*, Further, Ozerdem et al., Ellis et al., Sakurai et al., Otani et al. each provides evidence that supports Dr. Gerritsen’s testimony that “a skilled artisan would also conclude that neutralizing compounds capable of stimulating *c-fos* expression in pericytes (*e.g.*, PRO444) could be useful in preventing the onset and/or progression of cancer and/or angiogenesis.” Gerritsen Decl. ¶7. On the other hand, the Examiner has offered no significant reason or evidence to reject the Gerritsen Declaration, and, therefore, there is nothing in the record to controvert these statements of the Gerritsen declaration.

e. Conclusion - the Examiner’s arguments are not persuasive

In conclusion, Appellants have offered sufficient evidence to establish that it is more likely than not that one of skill in the art would believe that because PRO444 polypeptides induce *c-fos* in pericyte cells, PRO444 polypeptides are useful as targets for pericyte-associated tumors, and as stimulators of angiogenesis. This activity of the PRO444 polypeptide makes the antibody that specifically binds to it useful for purification of PRO444, and as a potential antagonist of PRO444. The references and declaration proffered by Appellants clearly support their asserted utility, and the Examiner has offered no relevant arguments or evidence to the contrary. In short, none of the Examiner’s responses to Appellants’ supporting evidence are sufficient to rebut Appellants’ asserted utility.

9. Utility – Conclusion

Appellants’ asserted utilities for the claimed antibodies as useful in the isolation of PRO444 polypeptides to be used as targets for therapeutics useful in the treatment of pericyte-associated tumors and as stimulators of angiogenesis correspond in scope to the subject matter sought to be patented and therefore “must be taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject.” *In re Langer*, 503 F.2d 1380, 1391, 183 U.S.P.Q. 288, 297 (C.C.P.A. 1974). The Examiner’s unsupported arguments and largely irrelevant references

Appl. No. : 10/066,273
Filed : February 1, 2002

are not sufficient evidence to make a *prima facie* showing that “one of ordinary skill in the art would reasonably doubt the asserted utility.” *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995).

Even if the Examiner has established a *prima facie* case, Appellants have offered sufficient rebuttal evidence in the form of expert declarations and references, which, when considered as a whole, establish that it is more likely than not that the asserted utility is true. *See In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992) (stating that the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or “more likely than not” standard); *M.P.E.P.* at § 2107.02, part VII (“evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true.”) (emphasis in original). Considering the evidence as a whole, the Board should find that Appellants have established at least one specific, substantial, and credible utility, and the Examiner’s rejection of Claims 40-44 under 35 U.S.C. §§ 101 as lacking utility should be reversed.

C. Enablement Rejection – Detailed Argument

The second issue before the Board is whether Appellants have enabled the pending claims such that one of skill in the art would be able to make and use the claimed invention. The Examiner has rejected pending Claims 40-44 under 35 U.S.C. §112, first paragraph, arguing that because the claimed invention is not supported by either a specific or substantial asserted utility or a well-established utility, one skilled in the art would not know how to use the claimed invention. *See final Office Action* at 9. For the reasons provided above, Appellants submit that Appellants have established at least one specific, substantial, and credible utility, and the Examiner’s rejection of Claims 40-44 under 35 U.S.C. § 112, first paragraph, as lacking utility should be reversed.

D. Conclusion

In view of the arguments presented above, Appellants submit that the specification as filed provides a specific, substantial and credible utility for the claimed antibodies, and, therefore, the claimed subject matter also is enabled. Appellants therefore respectfully request that the

Appl. No. : **10/066,273**
Filed : **February 1, 2002**

Board reverse the rejections of the pending claims as lacking utility under 35 U.S.C. §101, and as not being enabled under 35 U.S.C. §112, first paragraph.

Appl. No. : 10/066,273
Filed : February 1, 2002

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: April 26th, 2006

By: Anne Marie Kaiser
Anne Marie Kaiser
Registration No. 37,649
Attorney of Record
Customer No. 30,313
(619) 235-8550

Appl. No. : 10/066,273
Filed : February 1, 2002

VIII. APPENDIX A – CLAIMS ON APPEAL

40. An antibody that specifically binds to the polypeptide of SEQ ID NO:9.
41. The antibody of Claim 40 which is a monoclonal antibody.
42. The antibody of Claim 40 which is a humanized antibody.
43. The antibody of Claim 40 which is an antibody fragment.
44. The antibody of Claim 40 which is labeled.

Appl. No. : 10/066,273
Filed : February 1, 2002

IX. APPENDIX B – EVIDENCE

Attached hereto is a copy of the evidence cited in Appellants' Brief. The list of evidence below is accompanied by a statement setting forth where in the record that evidence was entered into the record by the Examiner.

Tab	Reference	Submitted	Entered
1	Kovács <i>et al.</i> (Neurochem. Int., (1998) 33:287-297)		Cited by the Examiner in the Office Action dated 4/28/04
2	Herrera <i>et al.</i> (Prog. Neurobiol., (1996) 50:83-107)		Cited by the Examiner in the Office Action dated 4/28/04
3	Janknecht <i>et al.</i> (Carcinogenesis, (1995) 3:443-450)		Cited by the Examiner in the Office Action dated 4/28/04
4	Saez <i>et al.</i> (Cell., (1995) 82(5):721-732)	Originally submitted with Appellants' Amendment and Response to Office Action mailed 7/27/04	Considered by the Examiner in the Office Action mailed 7/21/05
5	Marconcini <i>et al.</i> (Proc. Nat. Acad. Sci. USA (1999) 96(17):9671- 9676)	Originally submitted with Appellants' Amendment and Response to Office Action mailed 7/27/04	Considered by the Examiner in the Office Action mailed 7/21/05
6	Declaration by Mary Gerritsen, Ph.D.	Originally submitted with Appellants' Amendment and Response to Office Action mailed 1/18/05	Considered by Examiner in Office Action mailed 3/16/05
7	Coulon <i>et al.</i> (J. Biol. Chem. (1999) 274(43):30439-30446)		Cited by the Examiner in Office Action mailed 3/16/05
8	Sakurai <i>et al.</i> , (Invest. Ophthalmol. and Vis. Sci. (2002) 43(8):2774- 2781)		Cited by the Examiner in Office Action mailed 3/16/05
9	Otani <i>et al.</i> (Invest. Ophthalmol. and Vis. Sci. (2000) 41(5):1192- 1199)		Cited by the Examiner in Office Action mailed 3/16/05
10	Ozerdem <i>et al.</i> (Angiogenesis (2003) 6:241-249)		Cited by the Examiner in Office Action mailed 3/16/05

Appl. No. : 10/066,273
 Filed : February 1, 2002

11	McColl <i>et al.</i> , (APMIS (2004) 112:463-480)	Originally submitted with Appellants' Amendment and Response to Office Action mailed 6/15/05 as Exhibit A	Considered by Examiner in final Office Action mailed 7/21/05
12	Diaz-Florez <i>et al.</i> (Histol. Histopath. (1994) 9:807-843)		Cited by the Examiner in final Office Action mailed 7/21/05
13	Alon <i>et al.</i> (Nature Med. (1995) 1(10):1024-1028)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
14	Benjamin <i>et al.</i> (Proc. Nat. Acad. Sci. USA (1997) 94:8761-8766)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
15	Ellis, <i>et al.</i> (Oncology (2002) 16(5) Supp. 14-22)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
16	Ferrera <i>et al.</i> (Breast Cancer Res. Treat. (1995) 36:127-137)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
17	Fidler <i>et al.</i> (Cancer J. (2000) 6(Supp. 3):S225-236)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
18	Kirkpatrick, P. (Nature (2005) S8-S9)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
19	Kolch <i>et al.</i> (Breast Cancer Res. Treat. (1995) 36:139-155)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
20	Nehls <i>et al.</i> (Cell Tiss. Res. (1992) 270:469-474)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
21	Rhodin <i>et al.</i> (J. Submicrosc. Cytol. Pathol. (1989) 21(1):1-34)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
22	Shima, <i>et al.</i> (J. Biol. Chem. (1996) 271(7):3877-3883)	Originally submitted with Appellants' Submission with Request for Continued	Entered by Examiner in final Office Action mailed 11/25/05

Appl. No. : **10/066,273**
Filed : **February 1, 2002**

		Examination mailed 10/18/05	
23	Tischer, <i>et al.</i> (J. Biol. Chem. (1991) 266(18):11947-11954)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
24	Willett, <i>et al.</i> (Nat. Med. (2004) 10(2)145-147)	Originally submitted with Appellants' Submission with Request for Continued Examination mailed 10/18/05	Entered by Examiner in final Office Action mailed 11/25/05
25	Orlandini <i>et al.</i> (Proc. Nat. Acad. Sci. USA (1996) 93:11675-11780)		Cited by the Examiner in final Office Action mailed 11/25/05

• • • •
Appl. No. : 10/066,273
Filed : February 1, 2002

APPENDIX C – RELATED PROCEEDINGS

•
: There are no decisions rendered by a court or the Board in any related proceedings
: identified above.
•

2510927
040806

Invited review

c-Fos as a transcription factor: a stressful (re)view from a functional map

Krisztina J. Kovács*

Laboratory of Molecular Neuroendocrinology, Institute of Experimental Medicine, H-1450 Budapest, Hungary

Received 23 February 1998; accepted 6 April 1998

Abstract

This article summarizes the achievements that have been accumulated about the role of c-Fos as a transcription factor and as a functional marker of activated neurons. Since its discovery, more than a decade ago, as an inducible immediate-early gene encoding a transcription factor, or third messenger, involved in stimulus-transcription coupling and mediation of extracellular signals to long-term changes in cellular phenotype, c-fos became the most widely used powerful tool to delineate individual neurons as well as extended circuitries that are responsive to wide variety of external stimuli. There still remain uncertainties as to how general is the c-fos induction in the central neurons, and whether the threshold of c-fos induction is comparable along a certain neuronal circuit. The major limitation of this technology is that c-fos does not mark cells with a net inhibitory synaptic or transcriptional drive, and c-fos induction, as a generic marker of trans-synaptic activation, does not provide evidence for transcriptional activation of specific target genes in a certain cell type of interest. The first part of the review focuses on recent functional data on c-fos as transcription factor, while the second part discusses c-fos as a cellular marker of transcriptional activity in the stress-related circuitry. © 1998 Elsevier Science Ltd. All rights reserved.

Abbreviations:—ACTH, adrenocorticotrop hormone; ADX, adrenalectomy; AP-1, activator protein-1; ATF, activator transcription factor; AVP, arginine vasopressin; BNST, Bed Nucleus of Stria Terminalis; CaM, Calmodulin; cAMP, Adenosine 3'-5' cyclic monophosphate; CRE, Ca^{2+} /cAMP response element; CREB, cAMP response element binding protein; CRF, Corticotropin-releasing factor; FRA, Fos-related antigen; GAD, Glutamic acid decarboxylase; hnRNA, Heterogen nuclear RNA; HPA, Hypothalamo-Pituitary-Adrenocortical axis; IEG, Immediate-early Gene; IL-1, Interleukin-1; JNK, c-Jun N-terminal kinase; LH-RH, Luteinizing hormone-releasing hormone; LPS, (bacterial) Lipopolysaccharide; MAPK, Mitogen Activated Protein kinase; NGF, Nerve Growth Factor; NGFI-A,B, Nerve Growth Factor-induced proteins A and B; pCREB, CREB, phosphorylated at Ser¹³³; PDGF, Platelet-Derived Growth Factor; PENK, Proenkephalin; PKA/PKC, Protein kinases (A and C); PPI/PP2A, Protein Phosphatases (2A = calcineurin); PVN, Paraventricular Nucleus of the Hypothalamus; SAPK, Stress-activated Protein Kinase; SIE, SIS-inducible Element; SRE, Serum Response Element; SRF, Serum Response Factor; TCF, Ternary Complex Factor; TH, Tyrosine Hydroxylase; VSCC, Voltage Sensitive Calcium Channel.

1. Introduction

Stereotypic inducibility of c-fos proto-oncogene rendered this cellular immediate-early gene (IEG) to be the most widely used functional anatomical mapping tool to identify cells and extended circuitries that became activated in response to various stimuli (Greenberg and Ziff, 1984; Sagar et al., 1988; Ceccatelli et al., 1989; Bullit, 1990). On the other hand, c-fos (and other members of its family) by dimerization with that of the members of the jun family,

forms a transcription factor referred to as activator protein-1, AP-1 and transactivates other (late) genes subserving long-term changes in cellular phenotype. (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Most of those studies, however, that use c-Fos to map activated neurons do not focus on the mechanisms of how IEGs were activated and what the transcriptional consequence of their induction is. In contrast, recent achievements on the cellular/molecular mechanism of IEG induction and their effects on target gene regulation promote interpretation of c-fos maps. This paper summarizes these achievements and discusses functional implications of c-Fos strategy to map neuronal circuitries underlying stress-related pathways in the central nervous system.

*Tel.: 00 36 1 210 0819 ext. 135; fax: 00361 210 0811; e-mail: kovacs@koki.hu

Table 1
Some selected features of the Fos family of transcription factors

Protein	M [kDa]	Half-life	Gene	Features
c-Fos	55	2 h	c-fos	Rapid induction by acute challenges
FosB	46–48	9.5 h	fosB	Delayed induction by acute challenges
33Fos-like	33	10 h	fosB/ΔfosB	Induced by acute challenges
Fra-1	35	28 h	fosB/ΔfosB	In response to chronic/repeated stimuli
Fra-2	37	208 h	fosB/ΔfosB	In response to chronic/repeated stimuli

2. c-Fos as a transcription factor

2.1. *c-fos* and their relatives

To date four major protein members of the Fos family have been identified: c-Fos, FosB, Fra-1 and Fra-2. (Greenberg and Ziff, 1984; Zerial et al., 1989; Cohen and Curran, 1988; Nishina et al., 1990) (see Table 1.). These proteins are encoded by genes containing four exons and three introns (van Straaten et al., 1983) and possess a leucine zipper motif that promotes heterodimerization with that of the members of the Jun family (c-jun, JunB and JunD) to form a transcription factor referred to as activator protein-1 (AP-1) (for review see Hughes and Dragunow, 1995).

The gene *fosB* encodes a 46 kDa protein, FosB, and also has a splice variant called ΔFosB. ΔFosB cDNA gives rise to the 33, 35 and 37 kDa proteins, among which 35 and 37 kDa proteins correspond to chronic FRAs (Fos-related antigens) that are induced by chronic/repeated stimuli. The 33 kDa product is a Fos-like protein that is induced by acute treatments, as is the 55 kDa c-Fos protein encoded by the *c-fos* gene (Chen et al., 1997; Hiroi et al., 1997).

Fos and Jun family members undergo post-translational phosphorylation, mediated by Fos-regulatory kinase and c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase, SAPK) respectively, that affects their DNA binding abilities (Muller et al., 1987; Xu et al., 1997). The heterodimer AP-1 complexes interact with a consensus sequence TGACTCA in the regulatory regions of target genes and, depending on the composition of the AP-1 complex, stimulate or repress transcription (Rauscher et al., 1988). As c-Fos/c-Jun complexes have been reported to exert stimulatory effects on target gene expression, while c-Fos/JunB complexes are mostly inhibitory (Schüttler et al., 1989; Sheng and Greenberg, 1990), the sole reliance on induction pattern of

a single IEG in mapping studies does not provide any information about the transcriptional changes exerted by AP-1 in these cells.

Other members of the Fos family are constitutively expressed or, because of their extra-long half-lives, gradually accumulate in the cell nuclei with repeated stimuli to maintain the expression of the target genes or negatively feed back on the transcriptional activity. FRAs have been implicated in long-term phenotypical changes following chronic, tonic and repeated stimuli or neuronal disinhibition (Jacobson et al., 1990; Hoffman et al., 1993, 1994; Pennypacker et al., 1995; Hiroi et al., 1997).

2.2. Time domains of Fos induction

There are two features of c-fos expression that render this IEG as an excellent mapping tool: the low level of c-fos transcription under basal conditions and its inducibility upon wide range of trans-synaptic/transcriptional stimulation.

Under basal conditions, the detectable c-fos mRNA and protein levels are very low (Hughes et al., 1992). In PC12 cells, as well as in several brain regions in vivo, c-fos mRNA is induced within couple of minutes after acute challenge and peaks between 30 and 60 min. The maximal level of c-Fos protein occurs between 1 and 3 h, then it gradually disappears from the cell nucleus by 4–6 h after treatment (Sonnenberg et al., 1989a; Chan et al., 1993; Imaki et al., 1993; Ding et al., 1994; Ikeda et al., 1994; Cullinan et al., 1995; Kovács and Sawchenko, 1996). There are examples however, indicating either biphasic induction of c-fos expression following systemic interleukin challenge (Ericsson et al., 1994) and excitotoxic insults (Walker and Carlock, 1993) or acceleration of c-Fos response after adrenalectomy (Kovács and Sawchenko, 1997), that speak of the importance of performing time-course studies. Basal expression of FosB is a little higher, shows delayed activation and persists longer than c-Fos. The levels of FRAs rise with a delayed kinetics and gradually accumulate within the nucleus, especially after repeated/chronic stimuli (Morgan and Curran, 1989; Sonnenberg et al., 1989a; Honkainen et al., 1994). FRA staining might be exploited to study tonic activity and neuronal inhibition (Hoffman et al., 1993, 1994).

2.3. Threshold of Fos induction

It is generally thought that Fos induction reflects the functional activity of the neurons (Sagar et al., 1988; Dragunow and Faull, 1989; Duncan et al., 1993). In contrast, areas with high levels of neuronal activity (e.g. visual cortex (Kaczmarek and Chaudhuri, 1997); or magnocellular neurosecretory neurons during suckling (Fenelon et al., 1993) do not display significant c-fos expression, therefore, it seems that 'normal' neuronal

activity alone is not sufficient to induce immediate-early gene response. In line with this hypothesis, it has recently been shown that *fos* expression depends on the temporal features of action potential patterns. In cultured dorsal root ganglion cells, large and sustained increases in intracellular Ca^{2+} or high Ca^{2+} levels separated by long inter-burst intervals produced minimal *c-fos* expression. Immediate-early gene activation was inversely correlated with the burst-intervals of action potentials (Fields et al., 1997).

At systems level, 'usual' stimuli delivered by afferent inputs with the 'usual' intensity, do not result in any *c-fos* induction: sensory stimulation in the visual cortex results in *c-fos* expression only after a period of sensory deprivation, and when rats were exposed to novel objects (review: Kaczmarek and Chaudhuri, 1997). Auditory stimulation at different noise intensities revealed no *c-fos* induction under background noise situation but was activated by an intensity-dependent manner in auditory-related structures (Campeau and Watson, 1997).

It seems also likely that there is a threshold difference between different brain structures: some areas including basolateral, medial, and cortical nuclei of the amygdala, anteroventral and mediodorsal thalamus as well as cingulate, infralimbic and piriform cortices express *c-fos* in response to mild stimuli such as exploration of novel environment. These structures usually referred as constitutive *c-fos* expressing areas (Hughes et al., 1992; Cullinan et al., 1995; Duncan et al., 1995).

The effective interleukin-1 (IL-1) dose for eliciting *Fos* induction has been reported to be markedly different among the brain regions, circumventricular organs, the classically acknowledged sites involved in mediation of blood-borne signals displayed *c-Fos* only at the upper extremes of the dosages tested (Ericsson et al., 1994). The molecular mechanism underlying this spatial difference of *c-fos* inducibility is not known, but may reflect not only a different sensitivity of the signal transduction pathways but also might be due to the sensitivity of detection methods.

2.4. Mechanism of *fos* induction

There are a number of different challenges that induce *c-fos* expression: (1) neurotropic factors, (2) neurotransmitters, (3) depolarization and (4) increase of Ca^{2+} influx and elevation of intracellular/intranuclear Ca^{2+} (Greenberg and Ziff, 1984; Székely et al., 1987; Morgan and Curran, 1989; Doucet et al., 1990; Sheng et al., 1990; Sheng and Greenberg, 1990; Ghosh et al., 1994; Gaiddon et al., 1996).

Indeed, regulatory region of the *c-fos* gene contains several *cis*-acting elements (e.g. Ca^{2+} /CRE, SRE, SIS and AP-1, that could confer variety of *trans*-synaptic signals, commonly mediated via interdependent signal trans-

duction pathways, PKA, PKC, CAM kinase and MAP kinase cascades (Fig. 1.).

Increases of cAMP levels in the neurons result in *c-fos* activation via CRE (cAMP response element) mapped to -60 at the *c-fos* promoter (Sassone-Corsi et al., 1988; Sheng et al., 1990). The transcriptional activation is brought about by rapid phosphorylation of CREB (cAMP response element binding protein) via protein kinase A (Gonzalez and Montminy, 1989). CaM kinase cascade also phosphorylates CREB at Ser 133, and transduce changes in intracellular Ca^{2+} levels to immediate-early gene transcription through the same CRE (de Groot and Sassone-Corsi, 1993; Bito et al., 1996).

Growth factors induce *c-fos* expression through the serum response element, SRE, located at -300 from the transcription start site. This *cis*-acting element binds serum response factor (SRF) in association with TCF (ternary complex factor) containing Elk-1 or SAP-1 that are targets of the ras/MAPK (ERK) or PKC mediated phosphorylation (Treisman, 1992; Hill and Treisman, 1995).

c-fos gene possesses two, functionally distinct calcium detectors: Ca^{2+} influx through the L-type, voltage dependent Ca^{2+} channels (VSCC) induces CREB phosphorylation via CAM kinase pathway, and induces *c-fos* expression via CRE, while signals through the ligand gated Ca^{2+} channel (NMDA receptor) results in activation of the MAPK signal transduction pathway and targets SRE. (Bading et al., 1993; Ghosh et al., 1994). CRE and SRE may respond to spatially distinct pools of calcium: increases in nuclear calcium concentration control *c-fos* expression mediated via CRE, while SRE is triggered by a rise in cytoplasmic Ca^{2+} pool, and does not require an increase in nuclear Ca^{2+} (Hardingham et al., 1997; Santella and Carafoli, 1997). Thus, synaptic inputs that influence nuclear and cytoplasmic Ca^{2+} pools differently, may regulate distinct programs of gene expression. According to an exciting hypothesis (Ginty, 1997), *c-fos* gene expression may therefore be influenced by spatial aspects of Ca^{2+} signalling. Distal excitatory synapses that results in elevation of local, cytoplasmic Ca^{2+} pool, activate SRE-mediated gene expression, while proximal inputs that increase nuclear Ca^{2+} levels activate both CRE and SRE mediated transcription, and therefore increase the possibility of *c-fos* activation.

The *sis*-inducible element (SIE) has been proposed to contribute to *c-fos* induction by platelet-derived growth factor (PDGF) and gamma-interferon (Wagner et al., 1990; Robertson et al., 1995).

There is an AP-1 site in the *c-fos* promoter that lies adjacent to the SRE and binds Fos/Jun heterodimers as well as transcription factors of the CREB/ATF family and might mediate negative autofeedback for *fos* transcription (Sassone-Corsi et al., 1988).

Dephosphorylation of transcription factors could also be an important step regulating the persistence of *c-fos*

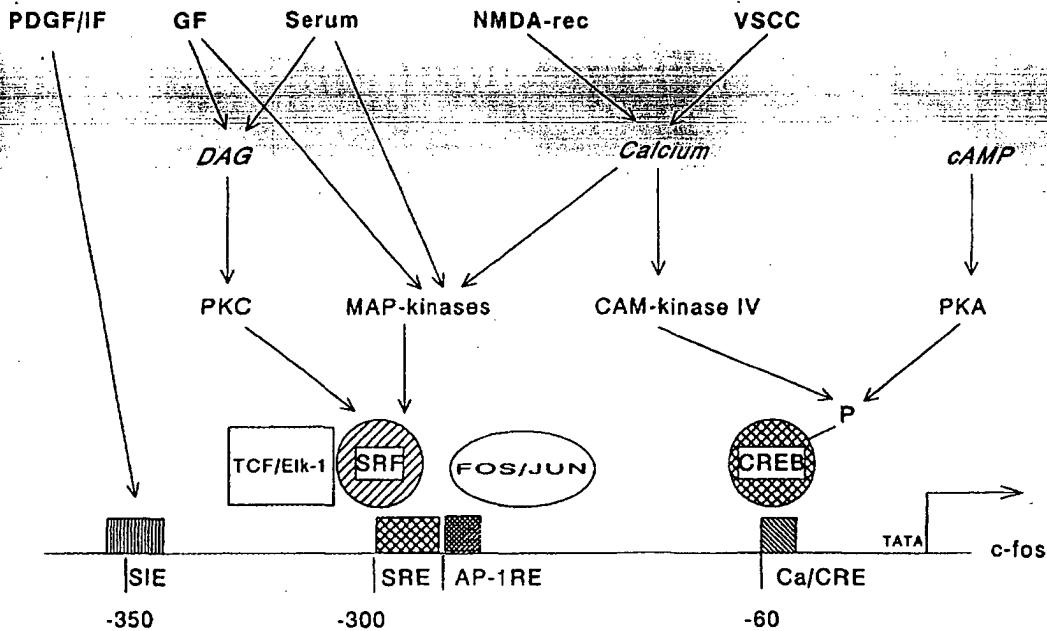


Fig. 1. Diagrammatic representation of the 5'-flanking regulatory region of the *c-fos* gene indicating the major upstream regulatory elements: the calcium-cAMP response element (Ca/CRE), the serum response element (SRE), the activator protein-1 binding site (AP-1RE) and the sis-inducible element (SIE). The transacting serum response factor (SRF), the ternary complex factor -Elk-1 (TCF/Elk-1) and the cAMP-response element binding protein (CREB) are targets of different signal transduction systems, including protein kinase A and C (PKA, PKC), calcium-calmodulin dependent kinases (CaM kinase) and mitogen activated kinases (MAP-kinase). Other abbreviations: PDGF, platelet-derived growth factor; GF, growth factors; VSCC, voltage-sensitive calcium channel.

expression. Phosphatase PP1 is responsible for the dephosphorylation of pCREB (Hagiwara et al., 1992), while TCF Elk-1 is dephosphorylated by PP2A, calcineurin (Hunter, 1995; Bito et al., 1996).

In contrast to the situation that seen in PC12 cells and in cultured neurons, data from intact animals indicated cross-talk between multiple interdependent control elements in regulation of *c-fos* in the central nervous system. CREB/CRE mediates neurotrophin responses via CaMK-dependent mechanism (Finkbeiner et al., 1997). Moreover, studies on mice carrying *fos-lacZ* transgene mutated at different individual regulatory elements, indicate that all regulatory elements are required in concert for tissue- and stimulus-specific regulation of the *c-fos* promoter (Robertson et al., 1995). Taken together, there are uncertainties as which signal transduction pathways and which regulatory elements mediate induction of *c-fos* expression in vivo mapping studies.

2.5. Fos/jun complex interaction with AP-1 binding site/gel-shift studies

To affect gene expression, protein products of *fos* family members should heterodimerize with that of *jun* family members and interact with AP-1 sites in the target genes. The interaction between transcription factors contained in whole cell/nuclear extracts with that of labeled oligonucleotides representing consensus sequences within the *cis*-acting response elements can be studied by gel

mobility shift assays, that indicate not only the relative binding affinities of nuclear factors to their DNA binding sites, but using specific antibodies in supershift reaction the composition of the transcription factors could also be revealed (e.g. Kaminska and Kaczmarek, 1993; Konradi et al., 1993; Pennypacker et al., 1994; Ogita and Yoneda, 1995). There are several combinations between *fos* and *jun* members. *c-Fos/c-Jun* complexes activate target gene expression, while *c-Fos/Jun B* heterodimer appear to be inhibitory (Sheng and Greenberg, 1990). Surprisingly, there is a considerable AP-1 binding affinity under basal conditions that composed of FosB and JunD proteins (Kaczmarek and Chaudhuri, 1997). Upon stimulation the composition of AP-1 transcription factor changes dynamically: the first transient complexes formed are made of *c-Fos* and *c-Jun*, that is followed by FosB/Jun dimers with longer half life, that are replaced by AP-1 complexes contain FRAs and Jun (Morgan and Curran, 1989; Sonnenberg et al., 1989) to maintain transcriptional activation in response to chronic and repeated stimuli.

The picture became even more complex with the finding that Fos and Jun could also interact with other transcription factors like pCREB or nuclear (steroid) receptors, and these cross-family heterodimers bind to different consensus sequences (Hai and Curran, 1991; Lukasiuk and Kaczmarek, 1994; Unlap and Jope, 1994).

Glucocorticoid receptor and AP-1 can antagonize each others activities in vivo and in vitro, and similar inter-

action have been described between AP-1 and other nuclear receptors (Pfahl, 1988).

2.6. Genes affected by AP-1 transcription factor

In spite of the enormous amount of data accumulated using *c-fos* in functional mapping studies we are still lacking direct evidences as to which genes are affected by AP-1 transcription factors in those neurons that show *c-fos* induction *in vivo*. To date the most studied candidate for AP-1 regulation was the proenkephalin (PENK) gene (Sonnenberg et al., 1989b; Bacher et al., 1996). Although *c-fos* induction precedes PENK expression in the hippocampus (Bing et al., 1997), those cells that express *c-fos* in response to NMDA or kainate are not enkephalinergic (Guo et al., 1996). Moreover, Konradi et al. (1993) could not detect Fos in complexes bound to CRE-2, the key second messenger inducible element in the PENK promoter, and antisense oligonucleotide injections into the apomorphin-challenged striatum, did not alter neuropeptide expression involved in basal ganglia function (Dragunow et al., 1994). Another model gene of AP-1 regulation is the prodynorphin (Hughes and Dragunow, 1995). Antisense knock-down studies (Hunter et al., 1995) provided direct evidence for *c-Fos* in regulation of prodynorphin induction, although other reports did not confirm cellular co-localization of *c-fos* and prodynorphin after kindled seizures (Simonato et al., 1996). Blockade of protein synthesis by cycloheximide, prevented the dehydration-induced vasopressin transcription in the magnocellular (Ding et al., 1994) and stress-induced expression in the parvocellular neurosecretory neurons (Kovács et al., 1998) suggesting involvement of *de novo* synthesized transcription factors encoded by the immediate-early genes. Other targets for *fos/AP-1* regulation of gene expression are some neurotransmitter synthesizing enzyme genes like GAD or TH (Najilerahim et al., 1991; Nestler, 1992; Sabban et al., 1995). Genetical approaches, including *c-fos* and FosB knockouts (Wang et al., 1992; Brown et al., 1996; Hiroi et al., 1997), transgenics (Smeyne et al., 1992; Kasof et al., 1995; Robertson et al., 1995) as well as antisense oligonucleotide injections (e.g. Hooper et al., 1994) will define the functional importance of immediate-early gene targets *in vivo*.

Another interesting feature of immediate-early gene-regulated transcription is the association of *c-fos* expression with neuronal cell death. Both *fos* and *jun* family members are activated in response to various excitotoxic insults, ischemia and seizures. There are two phases of *c-fos* induction during NMDA agonist-induced excitotoxic cascade: the first, transient phase is associated with seizures and the second, delayed phase, that results in cytoplasmic expression of *c-fos-lacZ* transgene, is evident exclusively in the vulnerable regions (Walker and Carlock, 1993; Kasof et al., 1995). Here again, however,

we can not identify with certainty the AP-1 target genes, as these rarely be under the control of a single class of transcription factor.

3. Stress-related pathways as revealed by *c-fos* mapping strategy

3.1. *c-fos* induction in the paraventricular nucleus

One of the best output of the *c-fos* strategy is the functional anatomical mapping of neuronal circuits underlying neuronendocrine-, autonomic- and behavioral responses induced by stress. Exogenous and endogenous challenges activate the parvocellular neurosecretory neurons in the hypothalamic paraventricular nucleus to initiate stress cascade by delivering the two main corticotropin releasing factors, CRF-41 and arginine vasopressin into the hypophyseal portal circulation (review: see Antoni, 1986). As revealed by numerous IEG-based mapping studies, acute challenges that result in an activation of the hypothalamo-pituitary-adrenocortical (HPA) axis specifically target CRF-41 containing, stress-related subset of the parvocellular neurons (Ceccatelli et al., 1989; Sharp et al., 1991; Hamamura et al., 1992; Honkaniemi et al., 1994; Chan et al., 1993; Kovács and Sawchenko, 1996). *c-fos* gene is upregulated in these neurons within 30 min after acute stress, while the maximal protein induction was found between 1 and 3 h. Although previous evidence has implicated various forebrain regions, (Sawchenko, et al., 1996) as well as brainstem catecholaminergic (Cunningham and Sawchenko, 1988) and serotonergic (Calogero et al., 1990) inputs in stress-induced activation of CRF-secreting neurons, there are uncertainties about the specific inputs and receptor systems that mediate *c-fos* induction in response to distinct stress paradigms. Discrete lesions separating ascending medullary pathways to the PVN prevent *c-fos* induction following acute IL-1 challenge, suggesting the involvement of catecholaminergic neurotransmission in activation of *c-fos* expression (Ericsson et al., 1994). Similar lesions however, were not effective in reducing *c-Fos* induction in the PVN following salt loading (Kovács and Sawchenko, 1993; Sawchenko et al., 1996) or footshock (Li et al., 1996).

It is still not clear however, whether the depletion of neuropeptide stores at the median eminence upon cellular stimulation, triggers immediate-early gene expression in the parvocellular neurons, and how such a retrograde signaling is mediated. Recent study revealed that nerve growth factor (NGF) and its receptor TrkA retrogradely transported from the axon terminals to the cell nucleus to trigger phosphorylation of transcription factor CREB (Riccio et al., 1997). Rapid CREB phosphorylation, that precedes *c-fos* induction was also revealed in CRF-secreting parvocellular neurons following different acute

challenges as ether, handling or restraint (Kovács and Sawchenko, 1996; Légrádi et al., 1997).

Several other IEGs, including NGFI-A, NGFI-B and TIS11 have been identified in the neurosecretory neurons and show rapid and transient induction in response to various acute and chronic stimulation (Chan et al., 1993; Honkaniemi et al., 1994).

There is a clear, although ill-defined threshold of c-fos induction in the paraventricular neurosecretory neurons. Weak stressors, such as exploration of novel environment, elevated plus maze, air puff do not result significant c-Fos immunoreactivity in the CRF secreting neurons (Duncan et al., 1996).

Where the stimulus intensity can be experimentally controlled, there is an intensity-dependent increase in c-fos induction, that is correlated with the increases in plasma corticosterone, a reliable marker of HPA axis activation (Ericsson et al., 1994; Campeau and Watson, 1997).

As with other systems, c-fos is a marker of those cellular/trans-synaptic activations that exceed a certain threshold and changes in the cellular phenotype is necessary for cellular adaptation. Therefore, c-fos based mapping technology does not, or hardly reveals normal, basal cellular activities.

3.2. Effect of glucocorticoids on c-fos induction and AP-1 transactivation: molecular basis of steroid negative feedback at the paraventricular nucleus

Interestingly, hypophysectropic CRF neurons do not display sustained c-fos mRNA or peptide induction after adrenalectomy (Brown and Sawchenko, 1997), a manipulation that results persistent synthetic and secretory activity as well as changes in neuropeptide phenotype in these neurons (Kiss et al., 1984; Sawchenko et al., 1984). Instead, adrenalectomy resulted only in a transient increase of c-Fos immunoreactivity that peaked 3 h after steroid removal, that was replaced by Fos-like immunoreactivity corresponding to chronic FRAs (Jacobson et al., 1990). These FRAs together with the constitutively expressed members of the jun family may represent the increased AP-1 binding activity which is seen in the hypothalamic extracts of adrenalectomized (ADX) rats (Kovács and Sawchenko, 1997). In the chronically ADX rat, CRF neurons are capable of c-fos expression since animals subjected to an additional stimulation (hypertonic saline or ether) display a robust induction of c-Fos, but the timing of IEG induction is accelerated, compared to rats that respond to acute challenges with a peak of plasma corticosterone (Kovács and Sawchenko, 1997). These results again speak to the importance of time course studies in c-fos mapping, although even a very detailed time course do not mechanically reveal functional relatedness and connectivity within a neuronal circuitry.

c-fos and other transcription factors encoded by inducible IEGs in cooperation with glucocorticoid receptors, that represent a class of ligand-activated transcription factors, provide a background for molecular mechanism of transcriptional integration at cellular level. Parvocellular neurons are capable to express both stimulation-dependent inducible IEG proteins and the glucocorticoid receptor, which mediates negative feedback effect of the adrenal steroids on the transcriptional activity of the CRF-secreting neurons. Protein-protein interaction could modify target gene transcription via composite glucocorticoid responsive element (Diamond et al., 1990), and conversely, ligand-activated glucocorticoid receptor could interact with AP-1 transcription factor (Yang-Yen et al., 1990; Schüle et al., 1990) in a situation and time dependent manner.

In addition to the protein-protein interaction that affect transactivation of both transcription factors, high levels of circulating glucocorticoids inhibit stress-induced c-fos expression in the paraventricular nucleus, via a yet unknown mechanism (Wan et al., 1993; Kovács and Sawchenko, 1997).

3.3. AP-1 target genes in the paraventricular nucleus

Genes, encoding hypothalamic neuropeptides (e.g. vasopressin, enkephalin, dynorphin, somatostatin, cholecystokinin, LH-RH) or neurotransmitter synthesizing enzymes (e.g. TH, GAD) that possess AP-1 consensus sequence(s) in their regulatory region can be putative target of c-fos mediated gene expression in the paraventricular nucleus. As stress-induced increase of c-fos expression precedes CRF mRNA accumulation in the neurosecretory neurons, CRF has also been suggested as a potential target of AP-1 transcription factor (Imaki et al., 1992), although the gene does not contain canonical AP-1 binding site (Chan et al., 1993). Using intron-specific probes that hybridize with sequences within the primary transcript (Herman et al., 1992), a very rapid stress-induced upregulation of CRF gene expression has been revealed in the parvocellular neurons that precedes the appearance of c-Fos protein by 1–2 h and seems to be parallel with CREB phosphorylation rather than with immediate-early gene activation (Kovács and Sawchenko, 1996, 1997). In contrast, arginine vasopressin (AVP) hnRNA levels in the CRF secreting cells show contemporaneous activation with c-Fos protein, suggesting an involvement of AP-1 transcription factor in parvocellular AVP gene regulation (Kovács and Sawchenko, 1996). Indeed, treatment of rats with protein synthesis blocker cycloheximide prior to acute ether challenge significantly inhibited the stress-induced elevation of c-Fos protein and AVP hnRNA in the parvocellular (Kovács et al., 1998) as well as hyperosmotic-challenge induced AVP mRNA increases in the magnocellular neurosecretory neurons (Ding et al., 1994). Studies in enke-

phalin transgenic mice suggest, that CREB, but not c-Fos is involved in the stress-induced transactivation of proenkephalin gene in the hypothalamic neurosecretory neurons, although the proenkephalin gene activation occurs later than c-Fos protein, and the promoter region of the pre-proenkephalin gene contains functional AP-1 response element (Borsook et al., 1994; Konradi et al., 1994).

3.4. Outlook on the stress-related circuitries

Stress models generally divided into two categories: (1) systemic stressors that target homeostatic parameters (e.g. hemorrhage, hypoglycemia, hyperosmotic challenge, immune stimuli or endotoxic shock), and (2) neurogenic (psychological, or processed) stressors that rely on somatosensory/nociceptive inputs plus involve a distinct, affective emotional component (e.g. footshock, restraint, immobilization).

All of these stressors result in activation of c-fos

mRNA and c-Fos like immunoreactivity in various brain areas defining neuronal circuitry involved in stress response (see Table 2). There are, however, several potential limitations of the c-fos-based functional mapping: it is not clear, whether the capacity for c-fos expression is universal among central neurons and whether the activated neurons express the c-fos gene with comparable sensitivities.

Areas that show c-fos expression in response to a given stressful stimulus are commonly grouped into three categories: (1) areas showing increased activation after handling; (2) areas involved in conveying stressor-specific information to the stress-related pathway; and (3) regions that mediate stereotypic neuronendocrine-, autonomic- and behavioral responses to stress.

Regions that display c-fos mRNA or c-Fos protein to handling and novel environment, irrespective of the stressor involve some thalamic and amygdaloid nuclei, as well as cortical and subcortical regions, lateral septum, anterior BNST, hippocampus and periaqueductal grey.

Table 2
Summary of c-fos induction following emotional and physical challenges

Region	Restraint	Immobil.	Foot shock	Swim	Plus maze	Air puff	Aggression	Fear	Audio	Ether	Pain	Osmotic	IL-1	LPS
Frontal cortex	+	+	++	++	++	+/–	+/–	+	0	++	+	+		+
Cingulate cortex	++	++	++	+++	++	+/–	+	++	+++	++	++	0	+	+
Orbital cortex	+++	+	+	++	+	0	0	+	++	+/–		0		
Pyriform cortex	+++	++	+++	+++	++	0	+/–	++	++	+++	+	+	+	++
Lateral septum	++	++	+	++	++	+/–	++	+/–	++	++		+/–	0	+
BNST	+	++	+/–	+	+	+/–	+	+++	++	++	+	+/–	++	+
Septohypoth. nucl.	++	+	++	++	+	0	+	+	++	+		+/–		
Circumventricular o.	0	0	0	0	0	0	0	0	0	0	0	+++	++	++
Hypothalamus														
PVN parvocell.	++	+++	++	++	+/–	+/–	+	++	++	+++	++	++	++	++
PVN magnocell.	+	+/–	+/–	+	0	0				0		++	++	+
Supraoptic nucl.	0	+	+	+/0	0	0	+	+/–	0	0	+	+++	++	++
Dorsomedial nucl.	+	+	+		+	0	+	+/–	+	+		0		++
Ventromedial nucl.	+	+	0		+/–	0	+	+	0	+/–		0		
Amygdala														
Basolateral nucl.	+	+	++	+	+	0			+/–	+	++	+/–	0	
Central nucl.	+/–	+	+/–	+/–	+/–	0	+	++	0	+		0	++	++
Cortical nucl.	+	+	++	+++	+	0	0	0	+	0		0	0	
Medial nucl.	++	+	++	+++	+	+/–	++	++	+	+	+	+/–	0	
Hippocampus	+	++	+	+	+/–	0		+	+/–	0		0	+/–	0
Periaqueductal grey	+	+	++	+	+	+	++	+	+	+	++			+
Locus coeruleus	+	+	++	++	+	0	+	++	+	+	+++		0	++
Nucl. solitary tract	++	++	++	++	+/–	0	+			++	++	++	++	++
Ventrolateral medulla	+	+	++	+	+/–	0	+		+/–	+	++	+	++	+
References	a,b,c	d,†	e,f,g	b,h	i,†	i	j	j	k	l	m,n,o	n,p,q	r	s,†

† Stands for results from our laboratory (unpublished)

*Chen and Herbert (1995); *Cullinan et al. (1995); *Melia et al. (1994); *Imaki et al. (1993); *Li et al. (1996); *Pezzone et al. (1992); *Sawchenko et al. (1996); *Duncan et al. (1993); *Duncan et al. (1996); *Kollack-Walker et al. (1997); *Campeau and Watson (1997); *Kovács and Sawchenko (1996); *Bullit (1990); *Ceccatelli et al. (1989); *Palkovits et al. (1996); *Hamamura et al. (1992); *Sharp et al. (1991); *Ericsson et al. (1994); *Wan et al. (1993).

indicating arousal (Cullinan et al., 1995; Duncan et al., 1996; Campeau and Watson, 1997; Kollack-Walker et al., 1997). The threshold of c-fos induction in these areas seems to be low, and sometimes referred to as sites for constitutive c-fos expression.

Afferent pathways that mediate neuroendocrine stress effects are stressor specific, including medullary catecholaminergic cell groups in response to hemorrhage (Chan and Sawchenko, 1994), LPS (Wan et al., 1993), IL-1 (Ericsson et al., 1994), pain (Palkovits et al., 1997), osmosensitive cell groups of the lamina terminalis to hypertonic salt loading (Hamamura et al., 1992; Kovács and Sawchenko, 1993; Larsen and Mikkelsen, 1995), auditory related structures to noise stress (Campeau and Watson, 1997).

Intriguing similarities among the activity maps within distinct stress models reveal the stress-related neurocircuitry that generally includes the effector neurons in the hypothalamic paraventricular nucleus, cingulate cortex, lateral septum, septohypothalamic nucleus, medial preoptic area, bed nucleus of stria terminalis (BNST), central amygdala, dorsal raphe and locus coeruleus: areas facilitating HPA axis activity (Cullinan et al., 1995; Larsen and Mikkelsen, 1995; Duncan et al., 1996; Sawchenko et al., 1996; Campeau and Watson, 1997; Kollack-Walker et al., 1997).

Although challenges as distinct as neurogenic foot-shock and systemic IL-1 injection activate indistinguishable population of neurons in the hypothalamic PVN and medullary catecholaminergic afferent areas, recent findings revealed a differential dependence of hypothalamic and medullary Fos-induction on the integrity of the stress-related circuitry. Medullary knife cuts, separating PVN from their ascending projections originating in the lower brain stem significantly reduced c-Fos in the PVN after systemic stress, while leaving c-Fos activation intact in the medullary catecholaminergic neurons. Opposite effects were seen after foot shock. (Li et al., 1996), suggesting that activation in the medulla is secondary consequence of the challenge in the neurogenic stress paradigm.

Lesions separating neurosecretory cells in the hypothalamic paraventricular and supraoptic nuclei from their descending projections from structures associated with the lamina terminalis abolished the effect of chronic salt loading, but not that of systemic IL-1 injection on the CRF-secreting cells (Kovács and Sawchenko, 1993; Ericsson et al., 1994; Sawchenko et al., 1996). These results emphasize the validation of results on functional connectivities obtained from c-fos pattern by experimental testing.

Another interesting feature of c-fos mapping in stress-related circuitry is that conditioned stressors induce c-fos expression in various cortical and subcortical areas (Beck and Fibiger, 1995), but not in the CRF-secreting parvocellular neurons.

3.5. Activation of c-fos expression in response to chronic challenges

Pattern of c-fos induction following acute challenges reveals basic stress-related circuitry, similarities and differences of activity maps taken from repeatedly or chronically stressed animals -however- bear pathological significance. In response to repeated restraint, c-fos activation is markedly reduced in the parvocellular neurosecretory neurons in the PVN, in the medial amygdala (Chen and Herbert, 1995) as well as within the cortex, hippocampus septum and brain stem (Melia et al., 1994). Habituation of c-Fos expression is consistent with the desensitized adrenocorticotropin (ACTH) response to repeated stimuli.

Osmosensitive areas of the lamina terminalis as well as magnocellular neurosecretory neurons in the paraventricular and supraoptic nuclei display c-Fos immunoreactivity in chronically salt-loaded rats. Chronic hyperosmotic challenge results in upregulation of CRF mRNA in Fos positive neurons, and coordinately down-regulates of CRF expression in the parvocellular neurons, and this inhibitory event is not marked by c-Fos induction (Kovács and Sawchenko, 1993).

As it has been discussed previously, adrenalectomy that represents chronic stimulation to the parvocellular neurosecretory neurons, results only transient c-Fos induction that is followed by chronic FRAs to maintain cellular activation (Jacobson et al., 1989; Brown and Sawchenko, 1997).

4. Summary

c-fos-based functional mapping has been validated as a powerful technic to reveal activated neurons and characterize cell groups that may be associated with functional circuits in a situation specific manner, in spite of uncertainties the exact functional roles of c-Fos, as a transcription factor, in the central nervous system as well as about the signal transduction mechanisms that activate immediate-early gene induction in vivo.

References

- Antoni, F.A., 1986. Hypothalamic regulation of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Reviews* 7, 351–378.
- Bacher, B., Wang, X., Schulz, S., Höllt, V., 1996. Induction of pro-enkephalin gene expression in cultured bovine chromaffin cells is dependent on protein synthesis of AP-1 proteins. *Journal of Neurochemistry* 66, 2264–2271.
- Bading, H., Ginty, D.G., Greenberg, M.E., 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* 260, 181–186.
- Beck, C.H.M., Fibiger, H.C., 1995. Conditioned fear-induced changes

- in behavior and in the expression of the immediate early gene *c-fos*: with and without diazepam pretreatment. *Journal of Neuroscience* 15: 709–720.
- Ding, G., Wilson, B., Hudson, P., Jin, L., Feng, Z., Zhang, W., Bing, R., Hong, J.S.: 1997. Single dose of kainic acid elevates the levels of enkephalins and activator protein-1 transcription factors in the hippocampus for up to 1 year. *Proc. Natl. Acad. Sci. USA* 94, 9422–9427.
- Bito, H., Deisseroth, K., Tsien, R.W.: 1996. CREB phosphorylation and dephosphorylation: a Ca^{2+} and stimulus duration-dependent switch for hippocampal gene expression. *Cell* 87, 1203–1214.
- Borsook, D., Konradi, C., Falkowski, O., Comb, M., Hymann, S.E.: 1994. Molecular mechanism of stress-induced proenkephalin gene regulation: CREB interacts with the proenkephalin gene in the mouse hypothalamus and is phosphorylated in response to hyperosmolar stress. *Molecular Endocrinology* 8, 240–248.
- Brown, E.R., Sawchenko, P.E.: 1997. Hypophysiotropic CRF neurons display a sustained immediate-early gene response to chronic stress, but not to adrenalectomy. *Journal of Neuroendocrinology* 9, 307–316.
- Brown, J.R., Ye, H., Bronson, R.T., Dikkes, P., Greenberg, M.E.: 1996. A defect in nurturing in mice lacking the immediate-early gene *FosB*. *Cell* 86, 297–309.
- Bullit, E.: 1990. Expression of *c-fos*-like protein as a marker for neuronal activity following noxious stimulation in the rat. *Journal of Comparative Neurology* 319, 517–530.
- Calogero, A.E., Bagdy, G., Szemerédi, K., Tartaglia, M.E., Gold, P.W., Chrousos, G.P.: 1990. Mechanism of serotonin receptor agonist-induced activation of the hypothalamic-pituitary-adrenal axis in the rat. *Endocrinology* 126, 1888–1894.
- Campeau, S., Watson, S.J.: 1997. Neuroendocrine and behavioral responses and brain pattern of *c-fos* induction associated with audiogenic stress. *Journal of Neuroendocrinology* 9, 577–588.
- Ceccatelli, S., Villar, M.J., Goldstein, M., Hökfelt, T.: 1989. Expression of *c-fos* immunoreactivity in transmitter-characterized neurons after stress. *Proc. Natl. Acad. Sci. USA* 86, 9569–9573.
- Chan, R.K., Sawchenko, P.E.: 1994. Spatially and temporally differentiated patterns of *c-fos* expression in brainstem catecholaminergic cell groups induced by cardiovascular challenges in the rat. *Journal of Comparative Neurology* 348, 433–460.
- Chan, R.K.W., Brown, E.R., Ericsson, A., Kovács, K.J., Sawchenko, P.E.: 1993. A comparison of two immediate-early genes, *c-fos* and *NGFI-B*, as markers for functional activation in stress-related neuroendocrine circuitry. *Journal of Neuroscience* 13, 5126–5138.
- Chen, X., Herbert, J.: 1995. Regional changes in *c-fos* expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. *Neuroscience* 64, 675–685.
- Chen, J., Kelz, M.B., Hope, B.T., Nakabeppu, Y., Nestler, E.J.: 1997. Chronic *Fos*-related antigens: stable variants of *deltaFosB* induced in brain by chronic treatments. *Journal of Neuroscience* 17, 4933–4941.
- Cohen, D., Curran, T.: 1988. *fra-1*: a serum-inducible, cellular immediate-early gene that encodes a *Fos*-related antigen. *Molecular Cell Biology* 8, 2063–2069.
- Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H., Watson, S.J.: 1995. Pattern and time course of immediate-early gene expression in rat brain following acute stress. *Neuroscience* 64, 477–505.
- Cunningham, Jr.E.T., Sawchenko, P.E.: 1988. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *Journal of Comparative Neurology* 274, 60–76.
- de Groot, R.P., Sassone-Corsi, P.: 1993. Hormonal control of gene expression: multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators. *Molecular Endocrinology* 7, 145–153.
- Diamond, M.I., Miner, J.N., Yoshinga, S.K., Yamamoto, K.R.: 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* 249, 1266–1272.
- Ding, J.M., Carver, W.C., Terracio, L., Buggy, J.: 1994. Proto-oncogene *c-fos* and the regulation of vasopressin gene expression during dehydration. *Molecular Brain Research* 21, 247–255.
- Doucet, J.P., Squinto, S.P., Bazan, N.G.: 1990. *Fos-Jun* and the primary genomic response in the nervous system. *Molecular Neurobiology* 27–55.
- Dragunow, M., Faull, R.L.: 1989. The use of *c-fos* as a metabolic marker in neuronal pathway. *J. Neurosci. Methods* 29, 261–265.
- Dragunow, M., Tse, C., Glass, M., Lawlor, P.: 1994. *c-fos* antisense reduces expression of *Krox-24* in rat caudate and neocortex. *Cellular and Molecular Neurobiology* 14, 395–405.
- Duncan, G.E., Johnson, K.B., Breese, G.R.: 1993. Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose and expression of *Fos*-like immunoreactivity. *Journal of Neuroscience* 13, 3932–3943.
- Duncan, G.E., Knapp, D.J., Breese, G.R.: 1996. Neuroanatomical characterization of *Fos* induction in rat behavioral models of anxiety. *Brain Research* 713, 79–91.
- Ericsson, A., Kovács, K.J., Sawchenko, P.E.: 1994. A functional anatomical analysis of central pathways subserving the effect of interleukin-1 on stress-related neuroendocrine neurons. *Journal of Neuroscience* 14, 897–913.
- Fenelon, V.S., Poulain, D.A., Theodosios, D.T.: 1993. Oxytocin neuron activation and *Fos* expression: a quantitative immunocytochemical analysis of the effect of lactation, parturition, osmotic and cardiovascular stimulation. *Neuroscience* 53, 77–89.
- Fields, R.D., Eshete, F., Stevens, B., Itoh, K.: 1997. Action potential-dependent regulation of gene expression: temporal specificity in Ca^{2+} , cAMP-responsive element binding proteins and mitogen-activated protein kinase signaling. *Journal of Neuroscience* 17, 7252–7266.
- Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., Greenberg, M.E.: 1997. CREB: a major mediator of neuronal neurotrophin responses. *Neuron* 19, 1031–1047.
- Gaiddon, C., Loeffler, J.P., Larmet, Y.: 1996. Brain-derived neurotrophic factor stimulates AP-1 and cyclic AMP-responsive element dependent transcriptional activity in central nervous system neurons. *Journal of Neurochemistry* 66, 2279–2286.
- Ghosh, A., Ginty, D.G., Bading, H., Greenberg, M.E.: 1994. Calcium regulation of gene expression in neuronal cells. *Journal of Neurobiology* 25, 294–303.
- Ginty, D.G.: 1997. Calcium regulation of gene expression: isn't that spatial? *Neuron* 18, 183–186.
- Gonzalez, G.A., Montminy, M.R.: 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59, 675–680.
- Greenberg, M.E., Ziff, E.B.: 1984. Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature* 311, 433–438.
- Guo, H.F., Cui, X., Hou, Y., Wang, X., Han, J.: 1996. *c-Fos* proteins are not involved in the activation of preproenkephalin gene expression in rat brain by peripheral electric stimulation (electroacupuncture). *Neuroscience Letters* 207, 163–166.
- Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., Montminy, M.R.: 1992. Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* 70, 105–113.
- Hai, T., Curran, T.: 1991. Cross-family dimerization of transcription factors *fos/jun* and *ATF/CREB* alters DNA-binding specificity. *Proc. Natl. Acad. Sci. USA* 88, 3720–3724.
- Hamamura, M., Nunez, D.J.R., Leng, G., Emson, P.C., Kiyama, H.: 1992. *c-fos* may code for a common transcription factor within the hypothalamic neural circuits involved in osmoregulation. *Brain Research* 572, 42–51.
- Hardingham, G.E., Chawla, S., Johnson, C.M., Bading, H.: 1997. Dis-

- tinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* 385, 260–265.
- Herman, J.P., Schafer, M.-H., Thomson, R.C., Watson, S.J., 1992. Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. *Molecular Endocrinology* 6, 1061–1069.
- Hill, C.S., Treisman, R., 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 80, 199–211.
- Hiroi, N., Brown, J.R., Haile, C.N., Ye, H., Greenberg, M.E., Nestler, E.J., 1997. FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc. Natl. Acad. Sci. USA* 94, 10397–10402.
- Hoffman, G.E., Smith, M.S., Verbalis, J.G., 1993. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Frontiers in Neuroendocrinology* 14, 173–213.
- Hoffman, G.E., Le, W.-W., Abbud, R., Lee, W.-S., Smith, S.M., 1994. Use of Fos-related antigens (FRAs) as markers of neuronal activity: FRA changes in dopamine neurons during proestrus, pregnancy and lactation. *Brain Research* 654, 207–215.
- Honkaniemi, J., Kononen, J., Kainu, T., Pyykönen, I., Peltö-Huikko, M., 1994. Induction of multiple immediate early genes in rat hypothalamic paraventricular nucleus after stress. *Molecular Brain Research* 25, 234–241.
- Hooper, M.L., Chiasson, B.J., Robertson, H.A., 1994. Infusion into the brain of an antisense oligonucleotide to the immediate-early gene c-fos suppresses production of fos and produces a behavioral effect. *Neuroscience* 63, 917–924.
- Hughes, P., Dragunow, M., 1995. Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacological Reviews* 47, 133–178.
- Hughes, P., Lawlor, P., Dragunow, M., 1992. Basal expression of Fos, Fos-related Jun and Krox 24 proteins in rat hippocampus. *Molecular Brain Research* 13, 355–357.
- Hunter, J.C., Woodburn, V.L., Durieux, C., Petterson, E.K., Poat, J.A., Hughes, J., 1995. c-fos antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. *Neuroscience* 65, 485–492.
- Hunter, T., 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80, 225–236.
- Ikeda, J., Nakajima, T., Osborne, O.C., Mies, G., Nowak, T.S.Jr., 1994. Coexpression of c-fos and hsp70 mRNAs in gerbil brain after ischemia: Induction threshold, distribution and time course evaluated by in situ hybridization. *Molecular Brain Research* 26, 249–258.
- Imaki, T., Shibasaki, T., Hotta, M., Demura, H., 1993. Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress. *Brain Research* 616, 114–125.
- Jacobson, L., Sharp, F.R., Dallman, M.F., 1990. Induction of fos-like immunoreactivity in hypothalamic corticotropin-releasing factor neurons after adrenalectomy in the rat. *Endocrinology* 126, 1709–1719.
- Kaczmarek, L., Chaudhuri, A., 1997. Sensory regulation of immediate-early gene expression in mammalian visual cortex: implications for functional mapping and neural plasticity. *Brain Research Reviews* 23, 237–256.
- Kaminska, B., Kaczmarek, L., 1993. Robust induction of AP-1 transcription factor DNA binding activity in the hippocampus of aged rats. *Neuroscience Letters* 153, 189–191.
- Kasof, G.M., Mandelzy, A., Maika, S.D., Hammer, R.E., Curran, T., Morgan, J.I., 1995. Kainic acid-induced neuronal death is associated with DNA damage and unique immediate-early gene response in c-fos-lacZ transgenic rats. *Journal of Neuroscience* 15, 4238–4249.
- Kiss, J.Z., Mezey, E., Skirboll, L., 1984. Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus become vasopressin positive after adrenalectomy. *Proc. Natl. Acad. Sci. USA* 81, 1854–1858.
- Kollack-Walker, S., Watson, S.J., Akil, H., 1997. Social stress in hamsters: defeat activates specific neurocircuits within the brain. *Journal of Neuroscience* 17, 8842–8855.
- Konradi, C., Cole, R.L., Heckers, S., Hyman, S.E., 1994. Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *Journal of Neuroscience* 14, 5623–5634.
- Konradi, C., Kobierski, L.A., Nguyen, T.V., Heckers, S., Hyman, S.E., 1993. The cAMP-response-element-binding protein interacts, but Fos protein does not interact, with the proenkephalin enhancer in rat striatum. *Proc. Natl. Acad. Sci. USA* 90, 7005–7009.
- Kovács, K.J., Sawchenko, P.E., 1993. Mediation of osmoregulatory influences on neuroendocrine corticotropin-releasing factor expression by the ventral lamina terminalis. *Proc. Natl. Acad. Sci. USA* 90, 7681–7685.
- Kovács, K.J., Sawchenko, P.E., 1996. Sequence of stress induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *Journal of Neuroscience* 16, 262–273.
- Kovács, K.J., Sawchenko, P.E., 1997. Glucocorticoid negative feedback is exerted selectively on vasopressin gene transcription in parvocellular neurosecretory neurons. *Soc. Neurosci. Abstr.* 27, Abstract # 797.8.
- Kovács, K.J., Arias, C., Sawchenko, P.E., 1998. Protein synthesis blockade differentially affects the stress-induced transcriptional activation of neuropeptide genes in parvocellular neurosecretory neurons. *Molecular Brain Research* 54, 85–91.
- Larsen, P.J., Mikkelsen, J.D., 1995. Functional identification of central afferent projections conveying information of acute stress to the hypothalamic paraventricular nucleus. *Journal of Neuroscience* 15, 2609–2627.
- Légrádi, G., Holzer, D., Kapcala, L.P., Lechan, R.M., 1997. Glucocorticoids inhibit stress-induced phosphorylation of CREB in corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. *Neuroendocrinology* 66, 86–97.
- Li, H.-Y., Ericsson, A., Sawchenko, P.E., 1996. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. *Proc. Natl. Acad. Sci. USA* 93, 2359–2364.
- Lukasiuk, K., Kaczmarek, L., 1994. AP-1 and CRE DNA binding activities in rat brain following pentylenetetrazole induced seizures. *Brain Research* 643, 227–233.
- Melia, K.R., Ryabinin, A.E., Schroeder, R., Bloom, F.E., Wilson, M.C., 1994. Induction and habituation of immediate-early gene expression in rat brain by acute and repeated restraint stress. *Journal of Neuroscience* 14, 5929–5938.
- Morgan, J.I., Curran, T., 1989. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *TINS* 12, 459–462.
- Morgan, J.I., Curran, T., 1991. Stimulus-transcription coupling in the nervous system: Involvement of the inducible proto-oncogenes fos and jun. *Annual Review of Neuroscience* 14, 421–451.
- Muller, R., Bravo, R., Muller, D., Kurz, C., Reiz, M., 1987. Different types of modification of c-fos and its associated protein p39: modulation of DNA binding by phosphorylation. *Oncogene Res.* 2, 19–32.
- Najlerahim, A., Showell, D.G.L., Pearson, R.C.A., 1991. Transient increase in glutamic acid decarboxylase mRNA in the cerebral cortex following focal cortical lesion in the rat. *Exp. Brain Research* 87, 113–118.
- Nestler, E.J., 1992. Molecular mechanisms of drug addiction. *Journal of Neuroscience* 12, 2439–2450.
- Nishina, H., Sato, H., Suzuki, T., Sato, N., Iba, H., 1990. Isolation and characterization of Fra-2, an additional member of the fos gene family. *Proc. Natl. Acad. Sci. USA* 87, 3619–3623.
- Ogita, K., Yoneda, Y., 1995. Detection of DNA binding activities of transcription factors with different protein motifs in nuclear extracts

- of murine brain by using gel-retardation electrophoresis. *Journal of Neurochemistry* 64, 1431-1439.
- Palkovits, M., Baffi, J.S., Dvornik, S., 1996. Neuronal organization of stress response: Pain-induced c-fos expression in brain stem catecholaminergic cell groups. *Annals New York Academy of Science* 77, 313-326.
- Pennypacker, K.R., Hong, J.-S., McMillan, M.K., 1994. Pharmacological regulation of AP-1 transcription factor DNA binding activity. *FASEB Journal* 8, 475-478.
- Pennypacker, K.R., Hong, J.-S., McMillan, M.K., 1995. Implication of prolonged expression of Fos-related antigens. *TIPS* 16, 317-321.
- Pezzone, M.A., Lee, W.-S., Hoffman, G.E., Rabin, B.S., 1992. Induction of c-fos immunoreactivity in the rat forebrain by conditioned and unconditioned stimuli. *Brain Res.* 597, 41-50.
- Pfahl, M., 1993. Nuclear receptor/AP-1 interaction. *Endocrine Reviews* 14, 651-658.
- Rauscher, F., Sambucetti, L., Curran, T., Distel, R., Spiegelman, B., 1988. Common DNA binding site for fos protein complexes and transcription factor AP-1. *Cell* 52, 471-480.
- Riccio, A., Pierchala, B.A., Ciarollo, C.L., Ginty, D.D., 1997. An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science* 277, 1097-1100.
- Robertson, L.M., Kerpola, T.K., Vendrell, M., Luk, D., Smeyne, R.J., Bocchiaro, C., Morgan, J.I., Curran, T., 1995. Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. *Neuron* 14, 241-252.
- Sabban, E.L., Hiremagalur, B., Nankova, B., Kvetnansky, R., 1995. Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes. *Annals New York Academy of Science* 771, 327-338.
- Sagar, S.M., Sharp, F.R., Curran, T., 1988. Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* 240, 1328-1331.
- Santella, L., Carafoli, E., 1997. Calcium signaling in the cell nucleus. *FASEB Journal* 11, 1091-1109.
- Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P., Verma, I.M., 1988. Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes and Development* 2, 1529-1538.
- Sawchenko, P.E., Swanson, L.W., Vale, W.W., 1984. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. *Proc. Natl. Acad. Sci. USA* 81, 1883-1886.
- Sawchenko, P.E., Brown, E.R., Chan, R.K.W., Ericsson, A., Li, H.-Y., Roland, B.L., Kovács, K.J., 1996. The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress. In: Holstege, G., Bandler, R., Saper, C.B. (Eds.), *Progress in Brain Research*. Elsevier Science, pp. 201-222.
- Schüle, R., Rangarajan, R., Klierer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M., Evans, R.M., 1990. Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. *Cell* 62, 1217-1226.
- Schüttle, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., Minna, J., 1989. jun-B inhibits and c-fos stimulates the transforming and transactivating activities of c-jun. *Cell* 59, 987-997.
- Sharp, F.R., Sagar, S.M., Hicks, K., Lowenstein, D., Hisanaga, K., 1991. c-fos mRNA, fos and fos-related antigen induction by hypertonic saline and stress. *Journal of Neuroscience* 11, 2321-2331.
- Sheng, M., Greenberg, M.E., 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* 4, 477-485.
- Sheng, M., McFadden, G., Greenberg, M.E., 1990. Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* 4, 571-582.
- Simonato, M., Bregola, G., Donatini, A., Bianchi, C., Beani, L., Ferri, S., Romualdi, P., 1996. Kindled seizure-induced c-fos and prodynorphin mRNA expressions are unrelated in the rat brain. *European Journal of Neuroscience* 8, 2064-2067.
- Smeyne, R.J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T., Morgan, J.I., 1992. fos-LacZ transgenic mice: mapping sites of gene induction in the central nervous system. *Neuron* 8, 13-23.
- Sonnenberg, J.L., Macgregor-Leon, P.F., Curran, T., Morgan, J.I., 1989a. Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. *Neuron* 3, 359-365.
- Sonnenberg, J.L., Rauscher, F., Morgan, J.I., Curran, T., 1989b. Regulation of proenkephalin by fos and jun. *Science* 246, 1622-1624.
- Székel, A.M., Barbaecia, M.L., Costa, E., 1987. Activation of specific glutamate receptor subtypes increases c-fos proto-oncogene expression in primary cultures of neonatal rat cerebellar granule cells. *Neuropharmacology* 26, 1779-1782.
- Treisman, R., 1992. The serum response element. *TIBS* 17, 423-426.
- Unlap, T., Jope, R.S., 1994. Dexamethasone attenuates kainate-induced AP-1 activation in rat brain. *Molecular Brain Research* 24, 275-282.
- van Straaten, F., Müller, R., Curran, T., van Beveren, C., Verma, I.M., 1983. Complete nucleotide sequence of a human c-onc gene: deduced amino acid sequence of the human c-fos protein. *Proc. Natl. Acad. Sci. USA* 80, 3183-3187.
- Wagner, B.J., Hayes, T.E., Hoban, C.J., Cochran, B.H., 1990. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. *EMBO Journal* 9, 4477-4484.
- Walker, P.D., Carlock, L.R., 1993. Immediate early gene activation during the initial phases of the excitotoxic cascade. *Journal of Neuroscience Research* 36, 588-595.
- Wan, W., Janz, L., Vriend, C.Y., Sorensen, C.M., Greenberg, A.H., Nance, D. M., 1993. Differential induction of c-Fos immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral administration of endotoxin. *Brain Research Bulletin* 32, 581-587.
- Wang, Z.-Q., Ovitt, C., Grigoriadis, A.E., Möhle-Steinlein, U., Rüther, U., Wagner, E.F., 1992. Bone and haematopoietic defects in mice lacking c-fos. *Nature* 360, 741-745.
- Xu, X., Raber, J., Yang, D., Su, B., Mucke, L., 1997. Dynamic regulation of c-Jun N-terminal kinase activity in mouse brain by environmental stimuli. *Proc. Natl. Acad. Sci. USA* 94, 12655-12660.
- Yang-Yen, H.F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T.J., Drouin, J., Karin, M., 1990. Transcriptional interference between cJun and glucocorticoid receptor: mutual inhibition of DNA-binding due to direct protein-protein interaction. *Cell* 62, 1205-1215.
- Zerial, M., Toschi, L., Ryseck, R., Schuermann, M., Müller, R., Bravo, R., 1989. The product of a novel growth activated gene, fosB, interacts with jun proteins enhancing their DNA binding affinity. *EMBO Journal* 8, 805-813.



PII: S0301-0082(96)00021-4

ACTIVATION OF *c-fos* IN THE BRAIN

DANIEL G. HERRERA*† and HAROLD A. ROBERTSON†

*Department of Neurology and Neuroscience, and Department of Psychiatry, The New York Hospital, Cornell Medical Center, 525 E 68th St (Box 147), New York, NY 10021, U.S.A. and

†Laboratory of Molecular Neurobiology, Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

(Received 18 March 1996)

Abstract—Activation of the proto-oncogene *c-fos* in the brain was described initially almost a decade ago and represents one of the most studied immediate early genes in the brain. Transient *c-fos* expression in the central nervous system was first observed after seizure activity and following noxious stimulation in the spinal cord. Since then, multiple studies have shown that different stimuli can induce *c-fos* expression. Seizure activity induces rapid and transient expression of *c-fos* in hippocampal structures. Similarly, transient activation of *c-fos* follows cortical brain injury in a pattern that resembles that of spreading depression. Many other stimuli have been shown to induce the expression of this proto-oncogene in the brain and *c-fos* immunostaining and *in situ* hybridization are now used to map brain metabolism under different physiological and non-physiological conditions. Here we review the variety of inducible patterns of *c-fos* expression in the brain. Copyright © 1996 Elsevier Science Ltd.

CONTENTS

1. Introduction	84
2. Immediate early genes	84
2.1. The <i>c-fos</i> proto-oncogene as an IEG	85
2.2. Fos-related antigens	85
2.3. <i>C-fos</i> in non-neural tissue	86
2.4. Activation of <i>c-Fos</i> <i>in vitro</i>	86
2.4.1. Neuronal cultures	86
2.4.2. Astrocytic cultures	89
3. Detection of <i>c-fos</i> and FRAs	89
4. The <i>c-fos</i> in the brain	89
4.1. Basal levels of <i>c-fos</i>	89
4.1.1. Adult non-human brains	89
4.1.2. The <i>c-fos</i> in human brain	89
4.1.2.1. Alzheimer's disease (AD)	90
4.2. Activation of <i>c-fos</i> following brain injury	90
4.2.1. Mechanical brain injury	90
4.2.2. Ischaemic brain injury	91
4.2.3. Heat shock	92
4.2.4. Spreading depression and NMDA receptors	92
4.3. Induction of <i>fos</i> after generalized seizures	93
4.3.1. Pentylentetrazol and kainic acid-induced seizures	93
4.3.2. Induction of <i>fos</i> by opiates and ethanol withdrawal	93
4.3.3. <i>Fos</i> and kindling	94
4.3.4. Electroconvulsive seizures and <i>c-fos</i> expression	95
4.3.5. Induction of <i>c-fos</i> by other convulsant agents	95
4.4. Learning and memory; relation to <i>c-fos</i> expression	96
4.4.1. <i>Fos</i> and long-term potentiation (LTP)	96
4.4.2. <i>C-fos</i> and learning	96
4.5. Neuroendocrine activation of <i>c-fos</i>	96
4.5.1. Osmotic stimulation	96
4.5.2. Stress	97
4.6. Circadian activation of <i>c-fos</i>	97
4.7. Pharmacological activation	98
4.7.1. Amphetamines, cocaine, dopamine agonists and antagonists	98
4.7.2. Activation of <i>c-fos</i> mediated by adrenergic receptors	99
4.7.3. Other pharmacological agents	99
4.8. Somatosensory and nociceptive stimulation of <i>c-fos</i> expression	100
5. Activation of <i>c-fos</i> during development	100
5.1. Basal levels	100
5.2. Seizures	101
5.3. Brain injury	101
6. Conclusions	101
Acknowledgements	101
References	101

† Author for correspondence.

ABBREVIATIONS

AD	Alzheimer's disease	L/D	Light/dark
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionate	LGN	Lateral geniculate nucleus
AON	Antisense oligonucleotide	LMT	Lamina terminalis
AP	Aminopurine	LTP	Long-term potentiation
CFLIR	<i>C-fos</i> -like immunoreactivity	MCA	Middle cerebral artery
CRF	Corticotropin releasing factor	NGF	Nerve growth factor
DG	Dentate gyrus	NMDA	<i>N</i> -methyl-D-aspartate
E	Embryonic day	NO	Nitric oxide
ECS	Electroconvulsive seizure	NOS	Nitric oxide synthetase
FBJ-MSV	FBJ-murine sarcoma virus	NRE	Negative regulatory element
FLIR	Fos-like immunoreactivity	nTG	Non-transgenic
FRA	Fos-related antigens	OHDA	Hydroxydopamine
GALV	Gibbon ape leukaemia virus	PD	Post-natal day
GFAP	Glial fibrillary acidic protein	PHE-I	Paired helical filament-I
GPDH	Glycerol-phosphate dehydrogenase	PMA	Phorbol-12-myristate-13-acetate
HS	Hypertonic solution	PTZ	Pentylenetetrazol
HSP	Heat shock protein	PVNm	Paraventricular nucleuspars magnocellularis
i.c.v.	Intracerebroventricular	PVNp	Paraventricular nucleuspars parvocellularis
IEG	Immediate early genes	QA	Quisqualic acid
i.p.	Intraperitoneal	SCN	Suprachiasmatic nucleus
IGL	Intergeniculate leaflet	SD	Spreading depression
IR	Immunoreactivity	SNC	Substantia nigrapars compacta
KA	Kainic acid	SNr	Substantia nigrapars reticularis
KCI	Potassium chloride	SO	Supraoptic nucleus
LC	Locus ceruleus	Tg	Transgenic
		TGI	Transient global ischaemia

1. INTRODUCTION

Neuroscience research in recent years has produced rapid and dramatic advances, especially in the area of molecular neurobiology. Montarolo *et al.* (1986) demonstrated that gene expression was required for the development of long-term facilitation in *Aplysia*, an adaptive behaviour that can be considered as a learning-related process in invertebrates. Some of the properties of nervous tissue include a capability for perceiving external changes and storing new information. It has been suggested that changes in gene expression could play a role in the storing of information and possibly in long-term memory (Goelet *et al.*, 1986), and that induction of genes by neuronal activity could result in long lasting changes.

The first set of genes activated by external signals are those that do not require *de novo* synthesis of proteins (immediate early genes or IEGs). The induction of IEGs is rapid and transient. Of these genes, probably the best studied is *c-fos*. The first studies that suggested which IEG expression could have a role in neuronal functioning were done on differentiated PC12 cells. Curran and Morgan (1985) showed the effects of NGF on *c-fos* expression in this cell line. Further studies demonstrated that depolarizing agents induced *c-fos* expression in neuronally differentiated PC12 cells (Morgan and Curran, 1986; Greenberg *et al.*, 1986). In 1987, Dragunow *et al.* (1987) showed the presence of basal levels of Fos-like immunoreactivity (FLIR) in rat brains. [Note: since specificity is an important issue in this review, we will refer to FLIR where it has not been established by Northern blotting that the antibody is selective for the 56 kDa c-Fos protein, and the term "*c-fos*-like immunoreactivity" (CFLIR) will be used when there is some degree of certainty that c-Fos protein is involved.] This report of the presence of FLIR in

brain was followed by three studies which demonstrated that increased neuronal activity *in vivo* could induce *c-fos* expression. The studies looked at *c-fos* induction after a generalized seizure (Morgan *et al.*, 1987); following noxious stimulation (Hunt *et al.*, 1987); and after electrical stimulation leading to kindling (Dragunow and Robertson, 1987a).

Since these pioneering studies, there have been hundreds of reports investigating *c-fos* expression in the central nervous system (CNS). Some investigators have used *c-fos* expression as a tool to study neuronal populations that are activated after different stimuli (Sagar *et al.*, 1988; Dragunow and Faull, 1989); this review will mainly focus on these studies as they pertain to the expression of *c-fos* in the brain.

2. IMMEDIATE EARLY GENES

Extrinsic signals can modulate cell function in different ways. External cues can affect cell function through the regulation of gene expression. Molecules that easily pass through membranes can directly modify gene expression by interacting with nuclear receptors. Substances that interact with receptors located on the cell membrane can alter the levels of second messengers and, subsequently, they can indirectly induce the expression of specific genes. Substances that cannot pass through membranes interact with receptors located on the cell surface and may induce a series of modifications within the cell. These changes may include: alterations in the intracellular concentration of second messengers which can modulate phosphorylation rates of different proteins, alterations in the influx of ions such as Ca^{2+} and modifications in ion channels. These are just a few of the possible changes that may

occur upon cell stimulation by extrinsic signals (Sheng and Greenberg, 1990).

Genes that are activated rapidly upon cell stimulation and whose expression cannot be prevented by protein synthesis inhibitors are the IEGs (Sheng and Greenberg, 1990). These IEGs are believed to encode, in most cases, transcription factors which will, in turn, modify the expression of other genes known as target genes (TG) (Sheng and Greenberg, 1990). Target gene expression will modify the phenotype of the cell in question. Thus, external signals can alter phenotypic expression of cells.

The list of IEGs expands every day; the most extensively studied, so far, are the proto-oncogenes *c-fos* and *c-jun*. Other IEGs have been identified such as those induced by NGF in PC12 cells; NGFI-A (Milbrandt, 1986) and NGFI-B (Milbrandt, 1987). The IEG NGFI-A also has been described as *egr-1* (Sukhtame *et al.*, 1988), *zif268* and *TIS-8* (Kujubu *et al.*, 1987; Lim *et al.*, 1987); NGFI-B is also known as *TIS-1* (Kujubu *et al.*, 1987; Lim *et al.*, 1987) and *N10* (Morgan and Curran, 1991; Nakabeppu and Nathans, 1991). Various adrenergic agonists, both α and β have been shown to upregulate most of the IEGs studied in astrocytes (Arenander *et al.*, 1989a, 1989b).

2.1. The *c-fos* Proto-Oncogene as an IEG

Proto-oncogene *c-fos* is the cellular homologue of the *v-fos* oncogene (Curran and Teich, 1982) and FBJ-MSV. The FBJ-MSV complex was isolated from a spontaneous osteosarcoma in a CF1 mouse (Curran *et al.*, 1984). The FBJ virus complex consist of a replication-competent helper murine leukaemia virus and a replication-defective transforming murine sarcoma virus (Curran *et al.*, 1984). The nucleotide sequence of the proviral DNA has been deduced (Van Beveren *et al.*, 1983), as has the sequence of the cellular progenitor of the *fos* gene from mouse and human cells (Van Beveren *et al.*, 1983; van Straaten *et al.*, 1983).

The major features of the organization of *fos* genes and its products are as follows (Verma, 1987).

Both *v-fos* and *c-fos* are basically homologous, except for four regions of non-homology, three of which represent introns with the appropriate splice donor or acceptor site. The fourth region represents sequences that have been deleted in the biogenesis of *v-fos*. This region is present both in mouse as well as in human *c-fos* genes (104 nucleotides). The c-Fos protein has 380 amino acids, just one less than the v-Fos protein. In the first 332 amino acids, murine c-Fos and v-Fos only differ in five residues, while the remaining 48 amino acids of the c-Fos protein are encoded in a different reading frame from that of the v-Fos protein. Thus, even though they are homologous, *c-fos* and *v-fos* have different carboxy terminals. Despite this fact, however, both proteins are located in the nucleus. The c-Fos protein undergoes more extensive post-translational modifications than v-Fos. Mouse and human c-Fos are homologous in 90% of their amino acid sequence, differing only in 24 out of 380 residues.

The structures of cellular and viral *fos* products and their association with a 39 000 Da cellular

protein (p39) were studied with an antisera raised against a synthetic peptide fragment of *c-fos* (Curran *et al.*, 1985). The p39 appears to form a complex only in the nucleus in a non-covalent manner (Sambucetti and Curran, 1986). Fos participates in the nuclear protein complex with a sequence element in a control region of the adipocyte (3T3-F442A) differentiation-sensitive gene, *aP2* (Franza *et al.*, 1988); this sequence, as noted by Franza *et al.* (1988) shares a common sequence with an enhancer element from the Gibbon ape leukaemia virus (GALV), and with a negative regulatory element (NRE) of the HIV-LTR (oligonucleotides -357 to -278). This common sequence element is remarkably similar to the AP-1 consensus binding site. A combination of structural and immunological comparisons has identified the Fos-associated protein p-39 as the protein product of the *jun* oncogene. Cell-surface stimulation can result in an increase of both *c-fos* and *c-jun* products (or other Jun family members like Jun-B). Thus, the products of these two proto-oncogenes and several related proteins induced by extracellular stimuli form a complex that associates with transcriptional control elements containing AP-1 sites, thereby potentially mediating long-term responses to signals that regulate growth and development (Franza *et al.*, 1988).

The products of *c-fos* and *c-jun* can interact synergistically, the *c-fos* product enhancing the binding of the c-Jun protein to DNA (Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988). The products of the nuclear proto-oncogenes, mentioned above, form a non-covalent association. These proteins, along with members of the *myc* family, share limited amino acid homology. A heptad repeat of leucine residues (the "leucine zipper") present within the region of homology may facilitate interactions between monomers of these proteins (Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988). The hydrophobic backbone generated by the leucine residues contacts the jun leucine repeat generating strong Van der Waal's-type bonds. Both proteins cooperate in binding to DNA. In conclusion, the Fos-Jun complex, due to this particular interaction, binds tighter to DNA than the Jun protein alone. The family of Jun (c-Jun-B, c-Jun-D, etc.) and Fos-related proteins (Fos-B, Fos-related antigens or FRA) makes possible a large number of combinations between members that should be able to interact with different portions of the genome (Sheng and Greenberg, 1990).

2.2. Fos-Related Antigens

Curran *et al.* (1985) produced polyclonal antibodies against c-Fos amino acids 127-152. This sequence is identical in v-Fos and c-Fos. This antibody precipitates Fos in the form of a protein complex with a set of cellular proteins (Curran *et al.*, 1985; Franza *et al.*, 1988). Within this set of proteins, there are several Fos-related antigens (FRAs) which cross-react with the c-Fos protein (Franza *et al.*, 1987). Up to 12 FRAs have been described (Franza *et al.*, 1987; Franza *et al.*, 1988). Several of these FRAs recognize, as does Fos, sequence elements that contain AP-1 binding sites (Curran *et al.*, 1985).

The FRAs can be induced in the brain by stimuli

that up-regulate *c-Fos* (Sonnenberg *et al.*, 1989). Since the initial study by Sonnenberg *et al.* (1989), several studies have shown a temporal or spatial dissociation in the induction of *c-fos* and Fos-related antigens (FRA) (Hoffman *et al.*, 1993; Earnest *et al.*, 1992; Zhang *et al.*, 1991, 1992b; Dragunow *et al.*, 1991a, 1991b, 1990d, 1990e; Aronin *et al.*, 1991; Sharp *et al.*, 1990, 1991; Hughes *et al.*, 1992; Anderson *et al.*, 1994). The expression of Fos and FRAs was analysed during postnatal development of the brain (Alcantara and Greenough, 1993). Transient Fos expression frequently was followed by a more protracted time course of FRA expression (Alcantara and Greenough, 1993). The FRAs were expressed in subplate neurons between postnatal day 1 (P1) and P15, in striatal striosome and matrix between P1 and P9, in entorhinal and piriform cortical neurons between P1 and P9, in cerebellar Purkinje neurons between P5 and P10 and in pyramidal neurons in the hippocampus between P1 and P9 (Alcantara and Greenough, 1993). The FRAs alone were expressed in pyramidal neurons in cerebral cortex between P1 and P15 (Alcantara and Greenough, 1993). In adult rats, low levels of FRAs IR were seen in the granule cells of the dentate gyrus (Hughes *et al.*, 1992) and in cerebellar Purkinje cells (Nakamura *et al.*, 1991).

2.3. *C-fos* in Non-Neural Tissue

Outside the CNS, *c-fos* localization often reflects the original association of the mouse viral oncogene, *v-fos*, with osteogenic sarcoma and bone formation. Thus, the transgenic mouse bearing a *c-fos* knockout lacks death and has bone deformations. Constant levels of *c-Fos* protein were observed in the undissected mouse placenta throughout the gestational period (Verma *et al.*, 1984). During mid-gestation, dissected placentae showed *c-fos* mRNA levels that were almost 15-fold higher in the outer portion of the placenta than in the differentiated syncytiotrophoblast. This suggests that, during mid-gestation, *c-Fos* protein originates mostly from maternally derived decidua basalis or from foetal cytotrophoblast. In the inner placenta, the levels of *c-Fos* protein increase gradually as gestation proceeds, reaching a level approximately 50% of that found in the outer portion (Muller *et al.*, 1982, 1984). Highest levels of *fos* expression were observed in the amnion (Verma *et al.*, 1984), almost as high as those observed in cells transformed by FBJ-MSV.

In the mouse foetus, the expression of *c-fos* was low throughout the gestational period from days 10 to 17; however, levels increased five-fold at later stages of prenatal development. Analyses of newborn tissues and 10-day-old mice revealed high levels of *c-fos* expression only in bone (rib cage and vertebra including bone marrow), muscles and part of the pleura and peritoneum subcutaneous tissue.

In *in situ* hybridization in mouse embryos, Dony and Gruss (1987) showed that *c-fos* expression was stage-specific and restricted to perichondrial growth regions of the cartilaginous skeleton. They also found high *c-fos* transcription in web-forming mesodermal cells with high growth capacity, suggesting a tissue-specific regulatory role of *c-fos* during

"differentiation-dependent growth" of foetal bone and mesodermal web tissue (Dony and Gruss, 1987).

Distribution of *c-Fos* in human tissues revealed a similar pattern to that described in mice; structure of the protein is highly conserved in humans when compared to the murine protein (van Straaten *et al.*, 1983). High levels of Fos protein were reported also in foetal liver. Among haematopoietic cells analysed, high levels of *c-Fos* had only been detected in mast cells, blood monocytes, differentiated neutrophils and macrophages. Several groups of cells show a low level of *c-Fos*, but *c-Fos* in these cells is highly inducible.

2.4. Activation of *c-fos* in Vitro

In most cell types, *c-Fos* protein levels are relatively low under basal conditions (Curran, 1988). However, serum and polypeptides can greatly stimulate, in a transient manner, *c-Fos* expression (Curran and Morgan, 1985; Greenberg *et al.*, 1986; Greenberg and Ziff, 1984; Sheng and Greenberg, 1990). Although these stimuli were linked to cell division, other signals associated with neuronal excitation also can elicit transient *c-Fos* expression, e.g. voltage-dependent Ca^{2+} channel activation (Greenberg *et al.*, 1986; Morgan and Curran, 1986) and neurotransmitters (Greenberg *et al.*, 1986). In PC12 cells, depolarizing stimuli might induce *c-Fos* through calmodulin and calmodulin-dependent proteins (Morgan and Curran, 1986). The protein kinase inhibitor 2-aminopurine (2AP) blocks the induction of the human β -interferon gene by poly I-poly C or by virus. In addition, it blocks the induction of *c-fos* by serum growth factors (Zinn *et al.*, 1988), suggesting that a protein kinase might be involved in the regulation of this gene.

2.4.1. Neuronal Cultures

Exposure of primary neuronal cultures to depolarizing stimuli induces the expression of *c-fos*. Szekely *et al.* (1987, 1989, 1990) demonstrated that glutamate-induced activation of *c-fos* was mediated through NMDA receptors in rat cerebellar neurons. Similar findings were observed in primary cultures of hippocampal neurons. Studies in cortico-striatal neuronal cultures demonstrated an increase in *c-fos* mRNA elicited through activation of NMDA, AMPA and metabotropic excitatory amino acid receptors (Vacarino *et al.*, 1992). Treatment of neuronal cultures with high potassium concentrations, a non-specific depolarizing stimulus, induces the expression of *c-fos* (Didier *et al.*, 1989; Bading *et al.*, 1993; Ghosh *et al.*, 1994). The activation of *c-fos* is dependent on extracellular Ca^{2+} and can be inhibited by EGTA, a calcium chelator; L-type Ca^{2+} channel blockers; and, by calmidazolium and KN-62 a, calmodulin and CaM kinase inhibitors, respectively (Bading *et al.*, 1993). Activation of *c-fos* through glutamate was inhibited by EGTA but unaffected by calmidazolium or KN-62 suggesting that different depolarizing stimuli activate *c-fos* through different calcium signalling pathways (Bading *et al.*, 1993).

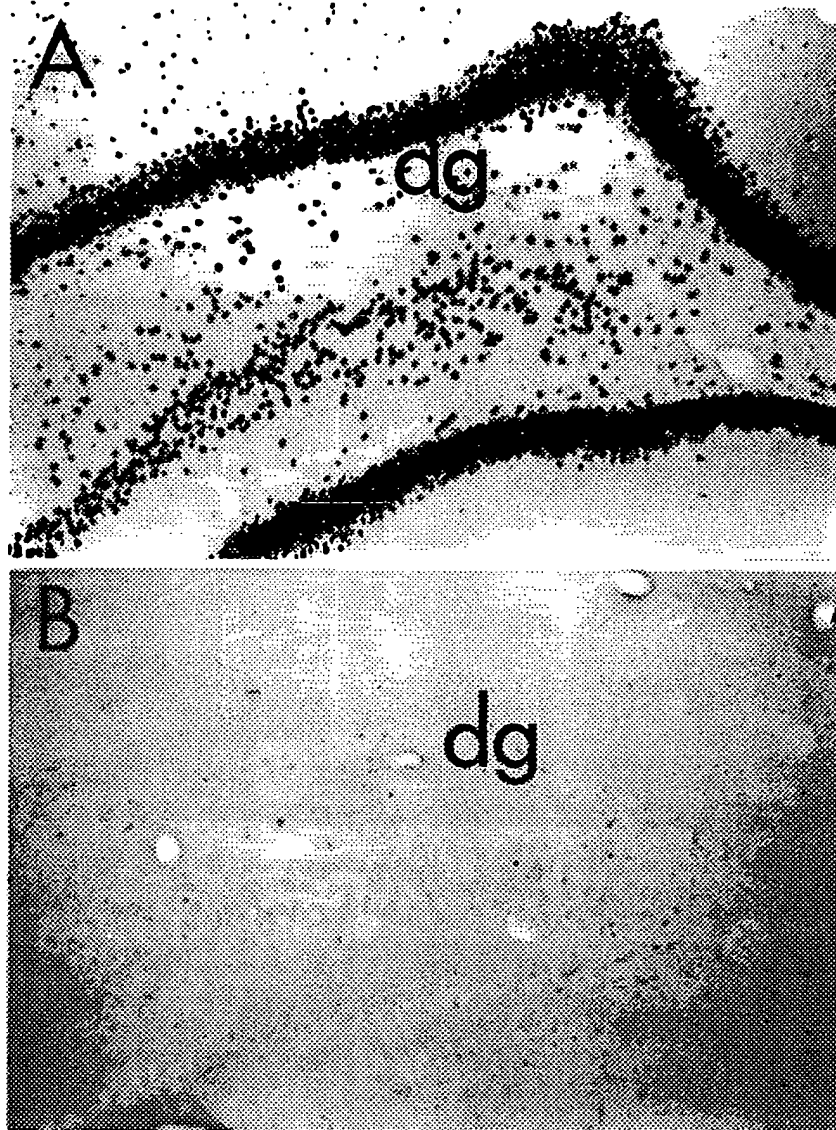


Fig. 1. Differential upregulation of *c-fos* after kainic acid-induced seizure. Administration of kainic acid (12 mg i.p.) results in the rapid induction of *c-fos*-like immunoreactivity. (A) Rat hippocampus 3 hr after KA administration. The animal was perfused intracardially with 4% paraformaldehyde, the brain was removed and processed for *c-fos* immunoreactivity (CRB sheep anti-*fos*). Note the intense nuclear immunostaining in the dentate gyrus (DG) of the hippocampal formation and in the pyramidal cell layers. (B) Administration of saline i.p. resulted in no increase in CFLIR when compared to controls (not shown).

Micrographs by Drs C. Plummer and H. A. Robertson.

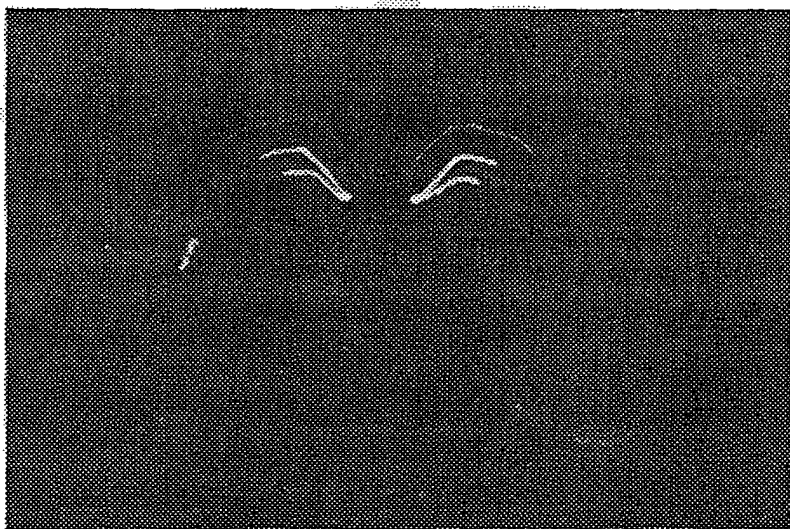


Fig. 2. *In situ* hybridization for *c-fos* 60 min after kainic acid administration. Note intense signal in the DG and in the pyramidal cell layers. The brain was removed and frozen, then sections were incubated with *c-fos* probe S35 for 14 days. Micrographs by Drs J. Armstrong and H. A. Robertson.

2.4.2. Astrocytic Cultures

Activation of *c-fos* in astrocytes *in vitro* has been demonstrated initially by Arenander *et al.* (1989a,b). These studies suggested that depolarizing stimuli, which induced *c-fos* in PC12 cells and neurons in culture, were ineffective in activating *c-fos* in astrocytes. High K⁺ concentrations (50 mM) did not activate IEGs. However, in a more recent study, higher K⁺ concentrations (140 mM) induced *c-fos* expression in a Ca²⁺-dependent manner (McNaughton and Hunt, 1992). Furthermore, *c-fos* expression was induced by excitatory amino acids. Unlike neurons, *c-fos* expression in astrocytes was not activated by *N*-methyl-D-aspartate (NMDA), which is expected given that astrocytes do not possess receptors for NMDA, but was induced by KA and quisqualic acid (QA) (Condorelli *et al.*, 1989; McNaughton and Hunt, 1992). The response to QA was mediated through both AMPA ionotropic receptor and QA metabotropic receptor (McNaughton and Hunt, 1992). Kainate receptor stimulation induced *c-fos* expression independent of extracellular calcium levels in contrast to neurons. Activation of *c-fos* in glial cells by excitatory amino acids appears to be an *in vitro* phenomenon (McNaughton and Hunt, 1992).

Induction of *c-fos* in astrocytic cultures is consistently elicited by trophic factors, phorbol-12-myristate-13-acetate (PMA).

3. DETECTION OF *C-fos* AND FRAS

The study of the activation of *c-fos* of the brain is obviously based on the detection of c-Fos protein (Fig. 1) or *c-fos* mRNA (Fig. 2). As discussed in Section 2.2, there are a set of proteins which share epitopes with Fos, the FRAs. These are induced by stimuli similar to those that upregulate *c-fos*. Thus, it is important, when looking at *c-fos* immunoreactivity, to consider which antibodies are being used and how specific they are for *c-fos*.

Among the antisera specific for *c-fos* only the following are included: 456 Fos (Medac-Genentechologie); Alu antiserum (raised against amino acids 1-131; Cohen and Curran, 1990); LA041 (Microbiological associates) raised against the N-terminal end, Fos 4-17; CRB OA-11-823 (Cambridge Research Biochemicals) raised against amino acids 2-16 of the N-terminal region of the c-Fos protein; PEPA 53 (Serotec) also raised against residues 2-16.

Within the antisera that recognize both Fos and FRAs, several studies were done; with the antisera raised against the M peptide (amino acids 127-152 of the Fos protein) by Curran *et al.* (1985), the commercially available PC05 Fos antibody and Abl (Oncogene Science) recognize FRAs.

4. THE *C-FOS* IN THE BRAIN

In this section, the expression of *c-fos* in the adult brain *in vivo* will be reviewed. Studies analysing the expression of *c-fos* under basal conditions will be considered initially. A recent paper had reviewed the expression of *c-fos* in central autonomic and sensory

systems (see Krukoff, 1993). In addition, a review on LTP and IEGs also is available (Abraham *et al.*, 1992).

4.1. Basal Levels of *c-fos*

Studies of the basal expression of *c-fos* are reviewed in a separate section on human brain studies. Within this latter section, recent studies on *c-fos* and Alzheimer's disease will be discussed. Further discussion on *c-fos* expression in the human brain can be found in Section 4.2.2.

4.1.1. Adult Non-Human Brains

The presence of *c-fos*-like immunoreactivity (IR) in the adult rat brain was initially reported in 1987 (Dragunow *et al.*, 1987). In that initial study, immunohistochemistry was done with polyclonal antibodies to the 27 amino acid fragment of the c-Fos protein known as M peptide (Curran *et al.*, 1985). Adult Sprague-Dawley rats were perfused intracardially with paraformaldehyde and the brains removed; a dark nuclear staining was found in hippocampal pyramidal neurons, granule cells of the dentate gyrus and in sparse neurons throughout the cerebral cortex (Dragunow *et al.*, 1987). In a more detailed study (Dragunow and Robertson, 1988a), using the same polyclonal antibodies, low levels of immunostaining were seen in areas CA1 and CA3 of the hippocampus, granule cells in the dentate gyrus as well as the amygdala, striatum, piriform cortex and cerebellum; Purkinje cells showed diffuse non-specific staining and staining in the midbrain, thalamus and septum was very low and absent in glial cells. It was suggested that the low levels of *c-fos* IR seen with the anti-M peptide did not necessarily mean the presence of basal levels of c-Fos protein, but might represent a cross-reacting protein(s).

A more recent study (Hughes *et al.*, 1992) reported the basal expression of the inducible IEG *fos*, *jun* and Krox-24 (previously known as TIS 8, *egr-1*, NGF-1A or *zif 268*) in the hippocampus. Antisera specific for (a) *fos* only; (b) *fos* and FRA; (c) Krox-24 and (d) Jun family (including c-Jun, Jun B and Jun D) were used. Very high basal levels of Jun were seen in the dentate granule cells with high basal levels of Krox-24 seen in the CA 1-subiculum region of the rat hippocampus. Basal levels of FRAs, but not of *c-fos*, were found in the dentate granule cells. These results strongly suggested a differential regulation in the expression of these IEGs and confirmed that basal levels of *c-fos* are very low.

4.1.2. The *c-fos* in Human Brain

The presence of FLIR was studied in sections from human anterior temporal cortex obtained from patients who were operated on for treatment of intractable partial complex seizures (Dragunow *et al.*, 1990c). The tissue was processed using antisera against the M peptide of the c-Fos protein which also detects FRAs. The FLIR was seen in the nucleus of neurons in the hippocampus, dentate gyrus and temporal cortex.

The CFLIR had been studied also in post-mortem

human brains. The IR was found in the cerebral cortex, hippocampus, striatum, thalamus and cerebellum, but not in the upper brain stem (Zhang *et al.*, 1992a).

4.1.2.1. Alzheimer's disease (AD)

The CFLIR or, more correctly, FLIR, has been sought in post-mortem in brains of patients with Alzheimer's disease. No staining was observed in the senile plaques or in neurofibrillary tangles, but there was a significant increase in FLIR in the hilus, the fimbria and in the CA1 area of the pyramidal cell layer (Zhang *et al.*, 1992a).

Recently, both Jun and FLIR were reported to be intensified in post-mortem brains of patients with Alzheimer's disease. Both Jun and FLIR were co-localized with paired helical filament-1 (PHF-1) in some neurons in AD brain (Anderson *et al.*, 1994). The PHF-1 is a marker for neurofibrillary tangles, one of the pathological hallmarks of AD. Furthermore, Jun and FLIR were co-localized with glial fibrillary acidic protein (GFAP)-positive astrocytes distributed in the cortex of AD and control cases, and in astrocytic cells surrounding thioflavine-stained plaques in AD brains. These findings suggested that *c-fos* and *jun* may play a role in the pathogenesis of AD, possibly reflecting the initiation of a cell death program in some neurons. Support for this hypothesis, came from a recent report by Smeyne *et al.*, 1993b), in which accumulation of *c-fos* was observed in the cytoplasm of fibroblasts undergoing apoptosis. Thus, a recent study (Rogaev *et al.*, 1993) observed the coding sequence of *c-fos* on chromosome 14 as a potential locus for familial AD; however, this hypothesis was not supported by the evidence found. The authors did not rule out a possible role for the non-coding sequences of *c-fos* in familial AD. In summary, although there is no evidence directly linking pathogenic mechanisms of AD to *c-fos* expression, a role for *c-fos* in AD cannot be ruled out. Although it seems certain that the *c-fos* locus is not involved in familial forms of AD (Morris and St. Claire, 1994), one of the possible loci for familial AD is on chromosome 14 at precisely the same site (14q: 23.1) as *c-fos* and *hsp-70*, the gene for the inducible heat shock protein (St-George-Hyslop *et al.*, 1992). However, both these genes have been excluded as the gene located at 14q: 23.1 (Morris and St. Claire, 1994; Rogaev *et al.*, 1993).

4.2. Activation of *c-fos* Following Brain Injury

In 1986, Verrier *et al.* (1986) demonstrated that wounding an *in vitro* fibroblast monolayer resulted in rapid and transient induction of the c-Fos protein. This induction was observed in the majority of cells lining the wound but not in those at a distance greater than approximately five cell layers from the wound. It has been shown previously (Stiles *et al.*, 1979) that quiescent BALB/c 3T3 fibroblasts may be induced to enter the cell cycle and reach S-phase not only when exposed to certain growth factors, but also when a confluent monolayer is wounded. These findings suggested that wounding in this system was equivalent to treatment with certain "competence"

factors. Thus, the fact that *c-fos* was induced after wounding of a monolayer led the authors to suggest an early role of *c-fos* in wound healing and tissue regeneration.

4.2.1. Mechanical Brain Injury

In the adult rat brain, Fos activation has been detected throughout the cortex after injury to this hemisphere from mechanical trauma (Dragunow and Robertson, 1988b). The ipsilateral cortex was affected throughout and no significant increase in FLIR was seen in the contralateral cortex. Increased FLIR was seen as early as 1 hr after the insult. Activation of *c-fos* appeared especially noticeable in cingulate and piriform cortices and was observed even 72 hr after the injury; however, maximal levels were attained within 6 and 12 hr. Immunohistochemistry for these experiments was done using an antibody directed at the M peptide which also detects FRAs (Dragunow and Robertson, 1988b).

Another remarkable finding in this study was the presence of *fos* immunoreactivity in glia-like cells. Maximal levels were visualized between 12 and 24 hr following brain damage. In a more recent report (Dragunow *et al.*, 1990a), double-labelling immunohistochemical studies demonstrated that FLIR could be detected in cells which also had positive GFAP immunoreactivity. However, *c-fos* IR was absent in cells which had glycerol-phosphate dehydrogenase (GPDH) IR (an oligodendrocyte marker). Importantly, no GPDH IR could be detected close to the injury. The FLIR was seen around the wound in non-neuronal cells that were both GFAP-positive and GFAP-negative. In a similar set of experiments following perforant-path lesion, which is accompanied by increase in GFAP IR in hippocampal astrocytes, *c-fos* IR was not seen in non-neuronal cells in the hippocampus, suggesting that induction of *c-fos* in glial cells could be seen after focal brain injury but not during Wallerian degeneration.

The fact that *c-fos* induction in these glia-like cells was limited only to the area surrounding the lesion suggests the possibility that *c-fos* in these cells is induced by factors diffusing from the damaged area. Therefore, from this initial study, it was evident that brain injury-induced *c-fos* activation was evident both at the lesion site and far from the lesion site throughout the cortex ipsilateral to the wound. The FLIR could be seen, as mentioned, up to 72 hr after injury (Dragunow and Robertson, 1988b; Sharp *et al.*, 1990; Sharp *et al.*, 1989). However, in a study in which immunohistochemistry was done with both a specific monoclonal antibody and with polyclonal antibodies against the M peptide, CFLIR could only be seen up to 72 hr after injury only with the polyclonal antibodies (Sharp *et al.*, 1990). The CFLIR (using the monoclonal, more specific, antibody) disappeared by 4 hr after cortical lesions. Furthermore, in that same study, *c-fos* mRNA, assessed by *in situ* hybridization, disappeared within 3–8 hr after cortical injury. A similar pattern of c-Fos expression was seen after administration of quinolinic acid into the lateral ventricles, suggesting an NMDA receptor-mediated process. Administration of CPP, a competitive NMDA antagonist, into the ventricles

prevented the induction of *c-Fos* in the ipsilateral cortex, except in the area of injury itself (Sharp *et al.*, 1990). The administration of quisqualic acid, a non-NMDA glutamate receptor agonist, induced CFLIR in the hippocampus but not in neocortex (Sharp *et al.*, 1990). Study of the temporal and spatial *c-fos* expression after unilateral cortical lesion using Northern Blot analysis and *in situ* hybridization showed a 20-fold increase in the *c-fos* mRNA content in the ipsilateral cortex and a more modest, yet significant, increase in the contralateral cortex (Ruzdijic *et al.*, 1993).

Cortical injections of nerve growth factor (NGF) were reported to induce a pattern of *c-fos* expression similar to the one described after traumatic cortical injury (Sharp *et al.*, 1989). It was hypothesized, at the time, that trophic factors or other molecules released at the site of the injury could contribute to *c-fos* induction throughout the ipsilateral cortex. However, it seems unlikely that simple diffusion of such factors will explain the pattern of immediate early gene expression.

Traumatic injury to the hippocampus induced *c-fos* expression throughout the dentate gyrus even far from the lesion without affecting the contralateral limbic system (Dragunow *et al.*, 1990b). Systemic administration (intraperitoneally) of MK-801, a non-competitive NMDA antagonist produced a dose- and time-dependent inhibition of *c-Fos* protein accumulation in neurons in the dentate gyrus and piriform cortex, without affecting expression of CFLIR in glial or ependymal cells (Dragunow *et al.*, 1990b).

Recently, the expression of mRNA for *c-fos* and the 72 kDa heat shock protein (HSP 72) was determined using *in situ* hybridization following lateral fluid-percussion injury (2.2–2.4 atm) in rat brain (Raghupathi *et al.*, 1995). In this model and, similarly to other models of brain injury, 2 hr after injury, induction of *c-fos* was observed throughout the cortex ipsilateral to the site of injury, while increased expression of HSP72 was observed only in cortical regions surrounding the contusion area; an increase in *c-fos* was observed bilaterally in the CA3 area of the hippocampus and the granule cells of the dentate gyrus and in the thalamus ipsilateral to the impact side (Raghupathi *et al.*, 1995). By 6 hr, increased expression of *c-fos* was observed only in the corpus callosum on the impact site. By 24 hr, *c-fos* mRNA had returned to control levels in all regions of the brain.

Mechanical injury to specific pathways within the CNS have been reported to affect the regulation of *c-fos* expression. Lesions, simultaneously produced to the entorhinal cortex and the fimbria-fornix pathway, both disrupting afferents to the hippocampus, result in a transient increase in *c-fos* expression in the dentate gyrus (DG) (Chen and Hillman, 1992). Dragunow (1992) showed that fimbria-fornix lesion induced Jun-like IR, but not *c-fos*, in medial septal-diagonal band neurons. He did not report induction of *c-fos* in the hippocampus. After medial forebrain bundle axotomy, *c-fos* expression was observed in the locus ceruleus and in the striatum, but not in the substantia nigra pars compacta where neurons would degenerate (Joh and

Weiser, 1993; Leah *et al.*, 1993; Weiser *et al.*, 1993). These results further support the idea that *c-fos* expression after injury may be needed for neuronal survival. Lesions of the SNc by 60HDA resulted in long-term overexpression of FRAs but not of *c-fos* (Dragunow *et al.*, 1991b).

4.2.2. Ischaemic Brain Injury

Studies of *c-fos* expression *in vivo* after ischaemic damage may be subdivided into those which investigate the effects of either global or focal ischaemia.

Transient global ischaemia after bilateral common carotid artery occlusion in gerbils (Ikeda *et al.*, 1990) induced the expression of *c-fos* mRNA in dentate gyrus neurons from 15 to 60 min after insult and in CA1 and CA3 neurons after 30–60 min. Studying *c-fos* mRNA using Northern blot analysis, Onodera *et al.* (1989) showed increased *c-fos* expression at 4 hr after a transient four-vessel occlusion in rats (Onodera *et al.*, 1989). In similar studies (Jorgensen *et al.*, 1989, 1991), increased expression of *c-fos* mRNA was observed 3 days after transient four-vessel occlusion in CA1 neurons of rat hippocampus. Analysis of *c-fos* and *c-jun* expression with *in situ* hybridization following transient forebrain ischaemia induced by four-vessel occlusion showed a pattern of activation most intense in the granule cells of the dentate gyrus (Wessel *et al.*, 1991). All animals underwent 20 min of transient ischaemia and highest activation was seen in the DG at 30 min after insult: hilar cells in the DG and CA3 pyramidal neurons showed a more delayed expression of IEG at about 60 min post-insult; neurons of CA1 exhibited a moderate signal at 1–2 hr post-insult. In the hippocampus, levels returned to baseline after approximately 6 hr. Similarly, granule cells in the cerebellum showed a rapid and transient increase in IEG expression after insult followed by a delayed expression in Purkinje cells with no further expression after 3 hr. Interestingly, as previously reported (Jorgensen *et al.*, 1989), there was a second peak of IEG activation in the CA1 pyramidal cells 24–72 hr post-insult. The CA1 pyramidal cells, along with small and moderate size spiny neurons in the dorsolateral striatum, undergo delayed neuronal death after transient global ischaemia. These results were in contrast with Northern blot analysis after forebrain ischaemia, which showed only a transient increase in *c-fos*, between 30 min and 4 hr. Uemura *et al.* (1991) reported increases in CFLIR in the hippocampus of gerbils after transient global ischaemia. The *c-fos* induction was seen in DG, CA3 and CA4 regions, 2–8 hr after transient bilateral carotid occlusion, which was prevented by the administration of MK-801 prior to the insult. There was no marked increase in *c-fos* IR in the CA1 area virtually at any time. The discrepancy could be secondary to the different species and techniques used; most likely, there is a dissociation between mRNA and protein synthesis in neurons that are undergoing delayed death. Expression of *c-fos* is induced strongly by influx of calcium (Morgan and Curran, 1986) which occurs at the time of neuronal death, and is inhibited by the Fos protein itself. Cell

death produces inhibition of protein synthesis, but the massive influx of Ca^{2+} that occurs with cell death may induce *c-fos* mRNA without the subsequent production of the c-Fos protein.

The patterns of CFLIR after transient global ischaemia in gerbils obtained by Bokesch *et al.* (1994) resembled those previously described by Uemura *et al.* (1991). In addition, it was found that dextrometorphan inhibited the induction of *c-fos* from 65 to 91% and protected against delayed neuronal death in the CA1 region of the hippocampus. Hyperglycaemia, which enhances brain injury from transient global ischaemia (TGI) was studied for its effect on *c-fos* expression post-TGI in gerbils (Combs *et al.*, 1992). Administration of glucose 4 g/kg prior to 20 min of bilateral carotid artery occlusion completely suppressed post-ischaemic *c-fos* expression (Combs *et al.*, 1992), suggesting that this effect could contribute to hyperglycaemia-enhanced ischaemic brain damage.

Levels of *c-fos* mRNA were studied after TGI of varied duration in gerbils (Ikeda *et al.*, 1994). Striking accumulation of *c-fos* mRNA was detected within 15 min of reperfusion in dentate granule cells, persisting through 1 hr, and a weaker signal was observed in CA1 and CA3 pyramidal neurons of hippocampus as well as in entorhinal cortex and neocortical regions in the same interval. Levels of *c-fos* mRNA had returned to control levels by 3 hr recirculation. Ischaemic insults of 1 min duration resulted in no detectable increase of *c-fos*, while 2 min ischaemia resulted in changes comparable to those seen after 5 min insults (Ikeda *et al.*, 1994). A recent study examined the temporospatial expression pattern of *c-fos*, *c-jun*, Jun-B and NGFI-B following 30 min of global ischaemia in rat brains using *in situ* hybridization and immunohistochemistry (Neumann-Haefelin *et al.*, 1994). Ischaemia caused a widespread early onset induction of *c-fos* and a late onset induction restricted to vulnerable regions. Late onset *c-fos* induction was observed in the CA1 region and the ventral thalamus but not in the striatum or neocortex, where neurons degenerate at a quicker pace (Neumann-Haefelin *et al.*, 1994).

Focal ischaemia, either by cortical devascularization or MCA occlusion, induces expression of *c-fos* in a pattern which resembles that observed after mechanical brain injury. There is increased *c-fos* expression throughout the cortex ipsilateral to the ischaemic injury, being especially marked in the piriform and cingulate cortices. Administration of NMDA antagonists prior to the injury (Herrera and Robertson, 1989, 1990a; Uemura *et al.*, 1991; Gass *et al.*, 1992; Christensen *et al.*, 1993) prevented the induction of *c-fos* in neurons but not in glial or ependymal cells. Neither atropine, a muscarinic receptor antagonist, calcium channel blockers (Herrera and Robertson, 1990a) or anti-inflammatory drugs (Olenik *et al.*, 1991), had any effect on the induction of *c-fos* after focal ischaemia.

In a recent study, An *et al.* (1993) confirmed several of these findings. The expression of not only c-Fos, but also Jun-B, c-Jun and Jun-D, were investigated in cerebral cortex after ligation of the MCA for 30 or 90 min. Expression of c-Fos and Jun-B followed the pattern previously described: gene up-regulation in

the ipsilateral cerebral cortex even far from the lesion site. The authors also reported increased c-Fos and Jun-B expression in the ipsilateral hippocampus. The anatomical distributions of c-Fos, Jun-B and, to a lesser extent, c-Jun, in these experiments were not explained by the authors, but it is likely that spreading depression (SD) may have played some role (see Herrera and Robertson, 1990a).

There seems to be an age dependency in the expression of c-Fos after hypoxia-ischaemia (Gunn *et al.*, 1990; Blumenfeld *et al.*, 1992; Munell *et al.*, 1994). After ischaemic brain damage, there was a more focal pattern of expression of HSP 72 compared to c-Fos (Welsh *et al.*, 1992; Ikeda *et al.*, 1994), suggesting different roles for both proteins.

The mechanisms underlying the induction of c-Fos after brain ischaemia are yet to be elucidated. To clarify the role of oxygen free radicals in the expression of c-Fos, the distribution of *c-fos* mRNA was investigated in Cu-Zn-superoxide dismutase transgenic mice (Tg) compared to control littermates (nTg) after a mild (10 min) focal cerebral ischaemia produced by transiently occluding the middle cerebral artery (MCA) (Kamii *et al.*, 1994). There was induction of *c-fos* mRNA at 1 and 6 hr after reperfusion in the ipsilateral hippocampus and thalamus in Tg mice, but only at 1 hr in the same regions in nTg mice. These results suggested to the authors (Kamii *et al.*, 1994) that oxygen radicals may suppress the expression of *c-fos* in the hippocampus and thalamus following transient occlusion of the MCA.

4.2.3. Heat Shock

Exposure of animals to elevated temperature can induce a specific set of proteins in the brain; these proteins are known as heat shock proteins (HSP) (Brown, 1990). It has been postulated that HSP may have a protective role from stressors, both *in vitro* and *in vivo* (Brown, 1990). Administration of heat shock to rabbits induced HSPs in the brain, mostly in glial cells (Sprang and Brown, 1987). A similar treatment given to rats induced the expression of FLIR in glial cells, in a pattern comparable to that observed in rabbits for HSPs (Dragunow *et al.*, 1989b). A more recent study demonstrated that hyperthermic, heat-exposed rats had significantly more Fos-immunoreactive neurons in the median preoptic nucleus and in preoptic areas than control or cold-exposed animals (Scammell *et al.*, 1993). These latter results help to establish that neurons in the preoptic areas participate in thermoregulation.

4.2.4. Spreading Depression and NMDA Receptors

The pattern of *c-fos* activation with the entire cortex, or hippocampus, ipsilateral to the injury being affected without contralateral involvement, prompted the idea that the underlying mechanism of *c-fos* activation was due to SD. Spreading depression is a temporary depression of electrical activity which can be elicited by electrical, mechanical or chemical stimulation applied topically to the cerebral cortex (Leao, 1944). Significantly, SD remains within the stimulated hemisphere and is inhibited with a high

dose of the NMDA-receptor antagonist ketamine (Hernandez-Caceres *et al.*, 1987; Marranes *et al.*, 1988). Similarly, *c-fos* activation is restricted to the damaged hemisphere and, like SD, is prevented by ketamine and related compounds (Herrera and Robertson, 1989, 1990a, 1990b; Herrera *et al.*, 1993c). Mechanical brain injury of the cortex also will disrupt the pia-arachnoid blood vessels producing focal ischaemia to the underlying cortex. By producing a limited disruption of the pia-arachnoids without producing direct damage to the underlying cortex, the effects of focal ischaemia vs those of direct mechanical injury can be isolated. Thus, cortical devascularization can induce *c-fos* activation in a pattern similar to that observed after mechanical injury possibly through SD (Herrera and Robertson, 1989, 1990a, 1990b).

Topical application of KCl to the brain surface, a reliably and consistent way of eliciting SD, results in the expression of FLIR throughout the ipsilateral cortex. This effect is inhibited by the NMDA antagonist MK-801 (Herrera and Robertson, 1990; Herrera *et al.*, 1993b). The induction of other transcription factors by SD has been reported recently (Herdegen *et al.*, 1993).

Mechanical injury of the hippocampus (Dragunow and Faull, 1990) and administration of high potassium concentrations directly into the hippocampus resulted in a similar pattern of induction of *c-fos* (Herrera *et al.*, 1993b). The concentrations of KCl used in the latter study were similar to those shown to consistently induce SD (Szerb, 1991). Furthermore, the induction of *c-fos* was NMDA receptor-mediated and correlated with increase in glutamate, but not acetylcholine, release (Herrera *et al.*, 1993b). A recent report supports a role for NMDA receptors in mediating *c-fos* induction after brain injury (Amir *et al.*, 1994). However, in that same study, it was shown that *fos* induction was not reduced in lactating dams (days 7–9), post-partum or progesterone-treated males, somehow contradicting the hypothesis that suckling stimulation and progesterone have a direct effect at the NMDA receptor (Amir *et al.*, 1994).

Spreading depression induces CFLIR, not only in the cortex but in subcortical structures as well. Increase in CFLIR after SD was reported in the superficial laminae of the trigeminal nucleus caudalis (Moskowitz *et al.*, 1993), possibly via trigeminovascular mechanisms.

Thus, it should be kept in mind that overexpression of *c-fos* after mechanical, chemical or ischaemic brain injury can be due partially to SD.

4.3. Induction of *fos* after Generalized Seizures

Activation and detection of *c-fos* *in vivo* has been proposed as a method to map metabolically the brain after specific stimuli (Sagar *et al.*, 1988; Morgan *et al.*, 1987; Dragunow and Faull, 1989). Thus, in retrospect, it is not surprising that activation of *c-fos* was initially described after the administration of seizure-inducing stimuli (Dragunow and Robertson, 1987a; Morgan *et al.*, 1987).

4.3.1. *Pentylenetetrazol and Kainic Acid-Induced Seizures*

The most striking feature of *c-fos* expression in the CNS is the dramatic, rapid and transient increase in *c-fos* mRNA and c-Fos protein levels following specific stimuli. For example, pentylenetetrazol (PTZ) administration evokes generalized seizures that produce a massive induction of Fos protein(s) in the cingulate and piriform cortices as well as in granule cells of the dentate gyrus (Dragunow and Robertson, 1987a, 1987b; Morgan *et al.*, 1987). Maximal levels of *c-fos* expression were attained in granule cells of the dentate gyrus within 30–60 min, and in pyramidal cells within 3–4 hr. At approximately this time, immunostaining in both neocortex and caudate showed high levels of *fos* immunoreactivity (Dragunow and Robertson, 1987b; Morgan *et al.*, 1987). Northern blot analysis of *c-fos* and *c-jun* in rat hippocampi after PTZ administration showed a rapid increase in *c-fos* (within 30–60 min) with a more protracted elevation of *c-jun*. This increase in proto-oncogene mRNA was followed by a parallel elevation in c-Fos protein and in AP-1-like DNA binding activity. As shown by Morgan *et al.* not only c-Fos protein, but also FRAs were elevated, of which some were still present in the brain up to 17 hr after seizure (Sonnenberg *et al.*, 1989).

In studies using transgenic mice, the pattern of *c-fos* expression was different in animals that received PTZ from those that received kainic acid (KA) (Smeyne *et al.*, 1992a, 1992b). The KA-induced seizures produced *c-fos* expression in limbic structures but minimal expression in cerebral cortex, while administration of NMDA antagonists had little effect. The PTZ induced *c-fos* both in limbic areas as well as in neocortex; and NMDA antagonists blocked proto-oncogene expression entirely.

Administration of KA results in a rapid increase in *c-fos* expression (see Figs 1 and 2) (Popovici *et al.*, 1988; LeGalle LaSalle, 1988; Jorgensen *et al.*, 1991; Schreiber *et al.*, 1993). At 90 min after KA administration increase in CFLIR was seen in the dentate gyrus of the hippocampus, particularly in the temporal pole. Moderate labelling was seen in primary olfactory nucleus, entorhinal cortex, medial amygdaloid nucleus and the pyramidal layer of CA1 region. At later times (3–6 hs) after KA administration CFLIR expression increased as the epileptic activity propagated to other limbic areas. CFLIR increased not only in hippocampus but also in cingulate cortex, amygdala, reticular thalamic nucleus and in some non-limbic structures such as the striatum. At 12 hr after KA administration, CFLIR was seen in primary frontal cortex with now lower levels in limbic areas. Similar experiments showed that CFLIR was increased in the DG up to 5 hr and in the pyramidal cell layers CA3, CA4 and particularly CA1 up to 24 hr after KA administration (LeGalle LaSalle, 1988).

4.3.2. *Induction of fos by Opiates and Ethanol Withdrawal*

Ethanol withdrawal seizures produce increased *c-fos* mRNA (Northern blot analysis) in mouse brain

(Dave *et al.*, 1990); the greatest increases were seen in the hippocampus, with a 40-fold increase at the time of the seizure (5–8 hr after ethanol withdrawal). Hippocampal *c-fos* mRNA was still elevated at 4 hr after the seizure and returned to baseline at 24 hr. In neocortex, levels increased 10-fold and were back to baseline within 2 hr after the seizure. Levels of *fos* mRNA were increased to a lesser extent in the cerebellum. These investigators found no increase of *c-fos* mRNA in mice that did not undergo seizures. Morgan *et al.* (1992) described increase in *c-fos* mRNA after cessation of ethanol exposure for 7 days. The exposure to ethanol itself induced only a transient increase in *fos* mRNA at 2–4 hr. After cessation of ethanol exposure, whole-brain *c-fos* mRNA increased, peaking at around 8 hr. *In situ* hybridization showed that significant elevation in *c-fos* expression, at 8-hr post-ethanol, occurred in the piriform cortex and in the dentate gyrus of the hippocampus. At 6 and 8 hr post-ethanol (at the times most likely for seizures to occur), the authors reported no difference between the magnitude of *c-fos* mRNA production in animals that seized and those that did not seize. The NMDA antagonist MK-801 (10 mg/kg) blocked peak expression of *c-fos* mRNA.

Precipitation of opiate withdrawal seizures in rats led to from a two- to three-fold increase in *c-fos* mRNA levels in the locus ceruleus 1–2 hr after initiation of withdrawal. Similarly, *c-fos* expression increased in the amygdala, ventral tegmentum, nucleus accumbens, neostriatum and cerebral cortex but not in the hippocampus, dorsal raphe (Hayward *et al.*, 1990).

4.3.3. *Fos* and Kindling

Kindling is a permanent change in the CNS by which a repeatedly administered but initially subconvulsive stimuli, either chemical or electrical, results in a generalized seizure (Goddard *et al.*, 1969).

Two of the most salient features of kindling which deserve special attention are (i) the irreversibility of this phenomenon; and (ii) the anatomical specificity. Repetitive administration of subconvulsive electrical stimuli to the amygdala will lead to kindling after a relatively small number of stimulations (from six to 15) (Goddard *et al.*, 1969). If the same type of stimuli is administered to the striatum, the number of stimulations will be much higher (mean 74).

Dragunow and Robertson, 1987a) showed that electrically induced seizure activity, of the sort which can lead to kindling, rapidly and transiently increases c-Fos protein-like immunoreactivity in the nuclei of granule cells of the dentate gyrus. In this study, the kindling electrodes were unilaterally implanted in the dorsal hippocampus. Maximal levels of c-Fos protein were achieved between 30 and 60 min following the electrical stimulation of hippocampal electrodes. The number of cells showing Fos activation was almost back to basal levels 24 hr later. Immunostaining also was increased in CA1, CA3 and CA4 of the hippocampus and in piriform cortex. Carbamazepine, an antiepileptic drug, dramatically reduced the number of cells activated in dentate gyrus following the stimuli.

Shin *et al.* (1990) confirmed these results. Using Northern blot analysis, they showed a great increase of *c-fos* mRNA in the hippocampus following a kindling stimuli in the angular bundle.

Fully kindled animals which had been seizure-free for 1 week showed no apparent alteration in basal levels of Fos immunoreactivity compared with non-kindled animals (Dragunow *et al.*, 1988).

Amygdala-kindled rats, perfused 4 hr after undergoing a stage 5 seizure in the classification of Racine (1972), showed a massive bilateral induction of *c-fos* throughout the cerebral cortex, hippocampus, piriform and entorhinal cortices (Dragunow *et al.*, 1988). Clark *et al.* (1991) extended these findings studying amygdala-kindled animals with *in situ* hybridization. Animals at stages 1 and 2 of kindling showed *c-fos* expression in a unilateral cortical distribution, affecting temporal, perirhinal and parietal cortices and amygdala with no changes in the hippocampus. A second pattern at early stages of kindling consisted of no changes in cortical areas, but increased *c-fos* expression in the hippocampus and amygdala. The animals with increased *c-fos* expression in the hippocampus had after discharges that were longer in expression than those with cortical expression. In later stages of kindling, the induction of *c-fos* was bilateral involving hippocampal, cortical structures, and the amygdala in a pattern similar to that described by Dragunow *et al.* (1988).

It seems likely that c-Fos protein levels in the dentate gyrus only increase after a kindled generalized seizure if the rats have had secondary after discharges. Non-kindled rats that received electrical stimulation in the amygdala show Fos induction in the stimulated amygdala and in the ipsilateral piriform and entorhinal cortices (Dragunow *et al.*, 1988). Angular bundle stimulation-evoked after discharges would only elicit *c-fos* expression in the hippocampus if the duration of the AD was more than 30 sec (Shin *et al.*, 1990). Further studies have shown that similar induction in gene expression can be seen with other IEGs (Simonato *et al.*, 1991). After evoking AD of more than 30 sec duration in the hippocampus within the DG cells, NGFI-A increased most rapidly (5 min); *c-fos* upregulation was seen in 30 min; and, *c-jun* overexpression took 30 min to become significant. In the CA1 area, a barely detectable increase in *c-jun* expression contrasted with increase in *c-fos* expression within 30 min of the AD. There was also a difference observed in the rate of decline in the expression of these genes, *c-fos* returned to baseline within 30 min but *c-jun* and NGFI-A were still up-regulated after 8 hr.

However, increased *c-fos* immunoreactivity after kindling stimuli does not necessarily mean that its presence is mandatory for the establishment of kindling. We have shown (Herrera *et al.*, 1988) that W7, a calmodulin antagonist that blocks the induction of *c-fos in vitro* after a depolarizing stimuli (Morgan and Curran, 1986), retards the development of kindling. Jonec and Wasterlain (1979) retarded the development of amygdala kindling using protein synthesis inhibitors, suggesting that newly synthesized protein is needed for the establishment of kindling.

4.3.4. Electroconvulsive Seizures and *c-fos* Expression.

Electroconvulsive therapy is a mainstream treatment for specific psychiatric disorders, but the mechanism of its efficacy remains unclear. Several studies have examined the effect of electroconvulsive seizures (ECS) on the expression of *c-fos* (Winston *et al.*, 1990; D'Costa *et al.*, 1991; Shebab *et al.*, 1992; Hope *et al.*, 1994).

Both ECS and PTZ induce FLIR in the dentate gyrus of the hippocampus, caudal amygdala, parts of the cerebral cortex, the bed nucleus of the stria terminalis, thalamus, lateral parabrachial nucleus and the nucleus of the solitary tract. In other structures, such as the medial and rostral amygdala, the ventromedial hypothalamic nucleus, the peripeduncular area, the central grey, and parts of the pretectum and superior colliculus, FLIR was induced by ECS, but not by PTZ (Shebab *et al.*, 1992). In another study, the responsiveness of *c-fos* and *c-jun* to an acute "test" seizure was examined after administration of one or more ECS (Winston *et al.*, 1990). Four hours after a single ECS, the induction of *c-fos* mRNA by a test seizure was blocked, in agreement with findings by Morgan *et al.* (1987), but by 18 hr, the levels of *c-fos* mRNA could be reinduced by the test seizure. However, chronic daily ECS treatments resulted in a time-dependent decrease in the expression of *c-fos* mRNA in response to a test seizure administered 18 hr after the last daily ECS, with a maximal effect at 8–10 days after starting daily treatment. Furthermore, the authors found that basal levels of c-Fos protein were reduced after chronic ECS. Compared with the findings by Morgan *et al.* (1987), which show that induction of *c-fos* is subject to a refractory period following a single PTZ-induced seizure, this suggests that the mechanisms by which acute seizure and chronic ECS block the induction of *c-fos* may differ (Winston *et al.*, 1990).

Attenuation of the expression of the Fos protein in various brain regions after a single ECS occurs in ageing mice (D'Costa *et al.*, 1991). Examination of the expression of Fos protein in various brain regions after a single ECS in young, middle-aged and old mice showed a consistent increase in the number of Fos-immunostained neurons in the amygdala, hippocampus and cerebral cortex in all age groups. Peak values were observed at 1 hr after a single ECS followed by a steady decline thereafter. However, when age-groups were compared, it was noted that CFLIR was induced differentially 1 hr after ECS. Middle-aged mice, when compared to young animals, showed 40–46% less CFLIR in the amygdala and cortical areas with no significant difference in the hippocampus. In old mice, there were 58–62% less *c-fos* positive cells 1 hr after ECS than in young mice in all brain regions studied, including the hippocampus (D'Costa *et al.*, 1991).

4.3.5. Induction of *c-fos* by other Convulsant Agents

The effects of other convulsants on *c-fos* expression have been reported. Lindane or *t* hexachlorocyclo-

hexane, at a convulsive dose, induced *c-fos* expression in piriform, entorhinal and cingulate cortices, in the dentate gyrus, the CA1 region of the hippocampus, the amygdala and several thalamic nuclei. At a lower, sub-convulsive, dose, *c-fos* mRNA was mainly found in cerebral cortex (Vendrell *et al.*, 1992a, 1992b, 1992c). The time course of *c-fos* induction was similar to that seen after PTZ administration. Nifedipine (Vendrell *et al.*, 1992a, 1992b), but not the NMDA antagonist MK-801, blocked the induction of *c-fos*, suggesting a role for L-type Ca^{2+} channels in the expression of *c-fos* during seizures. Further support for this role comes from the administration of Bay K, an L-type voltage-dependent Ca^{2+} channel agonist, that induced *c-fos* after producing seizures. The pattern of *c-fos* induction was different from that seen with other convulsants. High levels of *c-fos* mRNA were detected in the caudate putamen, superior colliculi, medial geniculate nuclei, anterior pretectal nuclei, occipital and retrosplenial cortices but not in the hippocampi. Administration of NMDA showed particular high levels of *c-fos* in cortical areas which were prevented by MK-801 (Vendrell *et al.*, 1992a, 1992b).

A similar pattern of fos induction was seen after generalized seizures produced by different stimuli; for example, kainic acid i.c.v. (Dragunow and Robertson, 1987a; Popovici *et al.*, 1988), kindling (Dragunow and Robertson, 1987a; Popovici *et al.*, 1988), and cortical electrical stimulation followed by secondary generalization of seizure activity (Sagar *et al.*, 1988). White and Gall (1987) described a dramatic increase in the levels of *c-fos* mRNA (as measured with Northern blotting), after recurrent seizures induced by unilateral lesion of the dentate gyrus hilus. They measured *c-fos* mRNA contents in the hippocampus and entorhinal cortex contralateral to the lesion. However, one interesting aspect of their study was that they also measured the levels of mRNA for c-myc, c-H-ras, preproenkephalin, preprocholecystokinin and preproenkephalin Y. Increases in *c-fos* but not of c-H-ras or c-myc preceded the increase of preproenkephalin mRNA in entorhinal cortex and hippocampus. However, *c-fos* is not the only gene upregulated following a generalized seizure. Saffen *et al.* (1988) studied the *in vivo* expression of four different immediate early genes (IEGs) after seizures induced by the administration of the convulsants PTZ or picrotoxin. The genes studied were *c-fos*, *c-jun*, *jun-B* and *NGFI-A* (*c-fos*/268/*egr-1*/Krox-24/*TIS-8*). Northern blot analysis as well as *in situ* hybridization showed that seizure led to an increase in the expression of these genes which was rapid (peak levels achieved within 1 hr) and transient (baseline levels seen 2 hr after convulsions). Because seizures were accompanied by increased neurotransmitter release (Lehmann and Hamberger, 1983; Lehmann *et al.*, 1983, 1985, 1986), it was suggested that IEG expression could be modified *in vivo* by neurotransmitters. Sukhtame *et al.* (1988) described the rise of *egr-1* mRNA in mouse brain following PTZ administration; the kinetics of *egr-1* induction were similar to those observed with *c-fos*.

Recent studies showed that administration of the GABAa antagonist, bicuculline, induced IEG expression in a similar pattern in neocortex and in

heterotopic cortical grafts after generalized seizures (Bele *et al.*, 1994).

4.4. Learning and Memory; Relation to *c-fos* Expression

Synthesis of protein is needed in order to develop long-term changes such as kindling (Jones and Wasterlain, 1979) in the CNS. As one of the earliest IEGs studied, the role of *c-fos* in learning and memory has been more thoroughly investigated than other IEGs.

4.4.1. Fos and Long-Term Potentiation (LTP)

Short-lasting tetanic electrical stimulations are able to enhance synaptic efficacy for hours or days; this effect is called LTP (Bliss and Lomo, 1973). Long-term potentiation is easy and reliably induced in the hippocampus both *in vivo* and *in vitro* by brief high frequency stimulation of mono- and polysynaptic excitatory pathways. The onset takes place within minutes and lasts from hours to weeks in some animals. Therefore, LTP has been invoked as a model of learning and memory.

The proto-oncogene *c-fos*, because of its rapid and transient inducibility and to the nuclear location of its product, was proposed as a key gene in the storage of information in the CNS. Thus, it was felt that elicitation of LTP might lead to *c-fos* induction. However, in anaesthetized animals where LTP was induced by electrically stimulating the perforant pathways, and even though good LTP was obtained, no *c-fos* induction could be observed in the dentate (Douglas *et al.*, 1988). Recently, it has been suggested that induction of *c-fos* can be observed in unanesthetized animals when stimulated at a narrow range of high frequency (Dragunow *et al.*, 1989a). Cole *et al.* (1989) and Wisden *et al.* (1990) showed that Krox-24 (or NGFI-A), and not *c-fos*, is consistently up-regulated in LTP and blocked by the NMDA non-competitive antagonist MK-801.

Other IEGs which seem to be consistently upregulated with LTP are *c-fos* related genes, *c-jun*, Jun-B and Jun-D. Thus, it seems that in this important model of memory, *c-fos* is not involved; however, there appears to be a role for other IEGs (see Abraham *et al.*, 1992 for review).

4.4.2. *C-fos* and Learning

Activation of *c-fos* does not appear to be necessary for LTP; however, there is some evidence that *c-fos* may have a role in learning processes. Experience-dependent stimulation of young chicks produces elevation of *c-fos* mRNA in cerebral cortex (Anokhin *et al.*, 1991). This induction of *c-fos* was not related to stress, arousal or sensory stimulation alone.

Training rats to attain a foot-shock motivated brightness discrimination in a Y-maze results in an increase of *c-fos* mRNA in the hippocampus (Tischmeyer *et al.*, 1990); however, pseudotraining produced a similar response. Long-term training of two-way active avoidance reaction induces *c-fos* (Nikolaev *et al.*, 1992).

These studies suggest a relationship between *c-fos*

expression and learning. However, the role of *c-fos* in these processes remains unclear. This is an area where it is expected that studies using mice with a transgenic knockout of *c-fos* or studies using antisense oligodeoxynucleotides against *c-fos* might be expected to yield results.

4.5. Neuroendocrine Activation of *c-fos*

This section will review the effects of osmotic stimulation, stress on the expression of *c-fos*, mainly in hypothalamic structures. A discussion of the expression of *c-fos* in relation to reproductive functions can be found in a review by Krukoff (1993).

4.5.1. Osmotic Stimulation

Sagar *et al.* (1988) showed that water deprivation for 24 hr led to increase in CFLIR in the supraoptic (SO) and paraventricular nuclei (PVN), and both the parvo (p) and magnocellularis (m) divisions, of the hypothalamus. Similarly, administering i.p. hypertonic solutions (HS) induced *c-fos* mRNA in PVNm, PVNp, SON and the lamina terminalis (LMT). This occurred within 30 min, peaked at 30–60 min, and disappeared after 180 min. Immunostaining specific for *c-fos* peaked 1–2 hr after hypertonic saline administration and faded within 4–8 hr, even with constant stimulation; in contrast, CFLIR persisted for 1–24 hr.

Immobilization stress, HS injection or capsaicin treatment, resulted in *c-fos* IR in oxytocinergic neurons in the PVNp, ventrolateral medulla oblongata, nucleus of tractus solitarius and in the locus ceruleus, but handling or i.p. sham injections produced no change in IR. (Ceccatelli *et al.*, 1989).

Injection of isotonic saline resulted in induction of *c-fos* mRNA in PVNp, anterior hypothalamus, suprachiasmatic nucleus, cingulate gyrus, piriform cortex, granule cells of the DG, hippocampal pyramidal neurons, ventral lateral septum and in paraventricular nuclei of the thalamus among other structures. This pattern of *c-fos* expression was considered to be due to the stress of handling and the injection itself. However, this pattern was not reported with immunostaining (Sharp *et al.*, 1991).

Systemic administration of hypertonic solutions is followed by overexpression of *c-fos* in the PVN, SON, lamina terminalis, and median preoptic nucleus within 60–120 min after stimulation. The expression of *c-fos* returns to baseline within 4–8 hr. When efforts were made to identify which subpopulation of neurons were expressing *c-fos* in the hypothalamus, it was found that approximately 60% of oxytocinergic neurons in the PVN and more than 90% in the SON were expressing *c-fos* after hyperosmotic stimulation. (Giovannelli *et al.*, 1990, 1992). Up-regulation of *c-fos* expression is not significantly altered upon destruction of the subfornical organ, which is thought to have some role in the response to osmotic stimulation (Giovannelli *et al.*, 1990, 1992).

Several studies showed co-localization of *c-fos* and vasopressin after osmotic stimulation (Roberts *et al.*, 1993; Shen *et al.*, 1992; Kjaer *et al.*, 1994; Ding *et al.*, 1994). Ding *et al.* (1994) demonstrated that not every vasopressinergic neuron expressed *c-fos* and vice

versa. The *c-fos* immunostaining and *c-fos* mRNA (by *in situ* hybridization) was seen in magnocellular neurons in the SON, PVN, nucleus circularis and adjacent accessory groups, similar to that observed in previous studies. Vasopressin up-regulation (potentially a target gene for *c-fos*) occurred after osmotic stimulation and was reduced with cycloheximide, a protein synthesis inhibitor. This suggests that *c-fos* and co-regulated IEGs may play a role in the expression of vasopressin and other peptides in the hypothalamus (Roberts *et al.*, 1993; Shen *et al.*, 1992; Kjaer *et al.*, 1994; Ding *et al.*, 1994; Giovannelli *et al.*, 1990, 1992; Koibuchi *et al.*, 1991; Wan *et al.*, 1993; Rowe and Erskine, 1993; Meister *et al.*, 1990).

4.5.2. Stress

Intracerebroventricular injection of colchicine, immobilization stress, HS injection and capsaicin treatment (but not handling or i.p. sham injections) resulted in CFLIR in parvocellular neurons in the PVNp; many of these cells were immunoreactive to corticotropin releasing factor. The CFLIR was also seen in the ventrolateral medulla oblongata, in the nucleus of tractus solitarius, and in the locus ceruleus. Many of these cells expressed catecholamine-synthesizing enzymes (Ceccatelli *et al.*, 1989). In these studies, it was clear that different stressful stimuli can induce *c-fos* in different subpopulations of cells. For instance, only a few magnocellular neurons in the PVN were *c-fos* positive after colchicine administration or immobilization stress as opposed to the effect of HS administration. The expression of *c-fos* in the PVN after immobilization stress was confirmed by several groups (Imaki *et al.*, 1992, 1993; Kononen *et al.*, 1992; Covenas *et al.*, 1993; Chastrette *et al.*, 1991; Harbuz *et al.*, 1993).

Levels of *c-fos* mRNA were analysed after presentation of mild footshocks to rats and produced increased *c-fos* expression in the amygdala (studies were done with Northern blots) (Campeau *et al.*, 1991). Similarly, presentation of contextual cues associated with footshocks, resulted in *c-fos* up-regulation in the amygdala; thus, conditioned and unconditioned fear resulted in *c-fos* upregulation. Handling of the animals for the first time also was followed by *c-fos* up-regulation in the amygdala (Campeau *et al.*, 1991). Removing rats from a cage and exposing them to a tone was sufficient to cause increases in *c-fos* mRNA (studied with *in situ* hybridization) in several forebrain areas (Smith *et al.*, 1992). Acute footshock stress resulted in further increases in *c-fos* expression in the septum cingulate and endopiriform cortices and in the PVN (Smith *et al.*, 1992). However, neither conditioned, nor unconditioned, stressors induced *c-fos* in the PVN in animals previously exposed to footshock, even though corticosterone levels were elevated. Another area affected by stressors was the locus ceruleus (Smith *et al.*, 1992; Pezzone *et al.*, 1992). Within the brainstem, not surprisingly, many of the cells expressing *c-fos* were catecholaminergic neurons (Pezzone *et al.*, 1993). Furthermore, lesioning the LC led to a decrease in the number of *fos*-positive neurons in cortex after immobilization stress (Stone *et al.*, 1993) suggesting a possible role for

catecholamines in the activation of *c-fos* during stress.

Many of the cells which showed *c-fos* activation in the PVN were capable of secreting corticotropin releasing factor (CRF) (Covenas *et al.*, 1993; Imaki *et al.*, 1992; Hoffman *et al.*, 1991; Jacobson *et al.*, 1990; Veening *et al.*, 1993; Kjaer *et al.*, 1994; Harbuz *et al.*, 1993) or express glucocorticoid receptors (Kononen *et al.*, 1992). The role of corticosteroids and CRF in the activation of *c-fos* during stress has been further emphasized in studies on the effect of CRF administration on IEG expression. Thus, i.c.v. administration of CRF resulted in increased *c-fos* expression in the basal forebrain including ventrolateral septum, dorsal and medial parvocellular divisions of the PVN, central nucleus of the amygdala and dorsal bed nucleus of the stria terminalis (Arnold *et al.*, 1992). This effect was prevented by i.c.v. pre-administration of a competitive CRF antagonist. A similar pattern of *c-fos* activation was seen with immobilization stress; however, there was no activation of *c-fos* in the amygdala. The CRF antagonist had no effect on the stress-induced *c-fos* in the PVN or in the ventrolateral septum, even though activation of *c-fos* in these areas correlated with the levels of endogenous corticosterone.

The effects of stress on *c-fos* expression has been compared recently with those of endotoxin administration (Wan *et al.*, 1994; Wan *et al.*, 1993). Pharmacological characterization indicated that stress-induced *c-fos* expression in the PVN and SON was attenuated by the NMDA antagonist MK-801 but not by indomethacin (a prostaglandin synthesis inhibitor). Administration of endotoxins resulted in a pattern of activation of *c-fos* similar, but not identical, to that induced by stress (Wan *et al.*, 1993, 1994). Activation of *c-fos* was attenuated by the NMDA antagonist MK-801 and, in contrast with stress-induced, by indomethacin.

Thus, it appears that stress-induced *c-fos* activation is the result of a complex neurochemical interaction involving at least catecholamines, glutamate and CRF.

4.6. Circadian Activation of *c-fos*

The suprachiasmatic nucleus (SCN) in the hypothalamus receives information from the retina either directly or indirectly. Cycles of light/dark (L/D) synchronize circadian rhythms by altering neuronal activity in the SCN of the hypothalamus, providing a circadian pacemaker (Rusak *et al.*, 1992). Exposure to light during the dark phase of the L/D cycle, but not during the L phase, resulted in upregulation of *c-fos* in the SCN in rats and in hamsters (Schwartz *et al.*, 1994; Rusak *et al.*, 1992; Rea *et al.*, 1993; Abe and Rusak, 1992; Rea, 1992; Earnest *et al.*, 1990; Earnest *et al.*, 1992, 1993). The expression of *c-fos* was accompanied by increased expression in other IEGs such as NGF-1A. The SCN receives visual inputs directly through a retino-hypothalamic tract, and indirectly through a polysynaptic retino-geniculate-hypothalamic pathway. The latter involves the intergeniculate leaflet (IGL) and adjacent parts of the ventral lateral geniculate nucleus (vLGN). Cells of these regions showed no FLIR in animals killed

during the D phase; however, after exposure to light during D phase, some animals (5/10 hamsters and 2/2 rats) did show intense FLIR in the ILG and external portion of the vLGN. The dorsal area of the LGN which also receives afferents from the retina did not show FLIR (Rusak *et al.*, 1992).

The minimum level of illumination required to induce *c-fos* was the same as that needed to produce a phase shift in the hamster's circadian rhythm of activity (Kornhauser *et al.*, 1990). Furthermore, the photic stimulation of *c-fos* was unaffected in mice with a mutation which causes profound loss of photoreceptors but failed to affect circadian responses to light (Colwell and Foster, 1992; Colwell *et al.*, 1993). This, together with the fact that light-induced *c-fos* occurred only within the D phase, when photic phase shifting of activity occurs, suggested that *c-fos* could be a molecular component of the photic pathway for entrainment of circadian activity (Kornhauser *et al.*, 1990; Rusak *et al.*, 1992).

In addition to light, there are other stimuli which can phase-shift circadian rhythms. These include saline injections, exercise in a novel environment and benzodiazepines. When phase shift occurred during the L phase, no *c-fos* upregulation was seen in the SCN (Colwell *et al.*, 1993; Janik and Mrosovsky, 1992; Kilduff *et al.*, 1992; Mead *et al.*, 1992), but did occur in the IGL.

Light-mediated induction of *c-fos* is a circadian time specific response in the SCN but not in the IGL (Park *et al.*, 1993).

The neurotransmitters involved in the activation of *c-fos* after photic stimulation have not been elucidated completely. However, it has been reported that the NMDA antagonist MK-801 can attenuate the up-regulation of *c-fos* in the rostral SCN and ventrolateral portions of the caudal SCN, but not in a discrete area of the dorsolateral SCN (Abe *et al.*, 1991, 1992). A role for acetylcholine has been suggested in a recent study that showed that mecamylamine, a cholinergic antagonist, blocked light-induced FLIR in areas of the SCN of the hamster (Zhang *et al.*, 1993). Administration of cocaine to pregnant rats induced *c-fos* expression in the foetal, but not in the maternal, SCN. The effect was blocked partially by a D1-dopamine receptor antagonist suggesting a mechanism through which maternally administered psychotropic drugs could affect the development of the circadian system (Weaver *et al.*, 1992).

It remains unclear why the activation of *c-fos* in the SCN occurs only upon photic stimulation during the D phase but not during the L phase even though retinal illumination during either phase results in a similar increase in firing in neurons of the SCN. Furthermore, dark-adapted retinas which show no FLIR, when exposed to light, display FLIR predominantly in the inner nuclear layer and to a lesser extent in the ganglion cell layer of the retina (Sagar and Sharp, 1990). Thus, it is likely that a permissive factor(s) (or inhibitor) must be present (or absent) during the D phase allowing photic stimulation to result in *c-fos* expression in the SCN.

Diurnal variations in the expression of *c-fos* also occur in other areas of the brain. The pineal gland (Carter, 1993), hippocampus and caudate-putamen

appear to express more FLIR after the onset of darkness (Kononen *et al.*, 1990). The results reported in the hippocampus and caudate-putamen could represent FRA rather than *c-fos*, since basal levels of *c-fos* itself in these areas are almost non-existent. Circadian effects on basal and stressed induced CFLIR were examined in the paraventricular nucleus of the hypothalamus, the habenula and the posterior paraventricular nucleus of the thalamus (Chastrette *et al.*, 1991). Stress induced the increase in CFLIR in all three nuclei, independent of time but CFLIR in non-stressed animals was lower in the habenula during night-time.

4.7. Pharmacological Activation

This section will discuss the role of different neurotransmitter systems in the activation of *c-fos*, specifically the role of catecholaminergic systems. The participation of glutamatergic systems are also considered, but further discussion on this topic can be found in Sections 4.2.1–4.2.3.

4.7.1. Amphetamines, Cocaine, Dopamine Agonists and Antagonists

Unilateral lesion of dopaminergic neurons in the ventral mesencephalon with 6-OHDA results in a model of unilateral Parkinsonism. When lesioned animals are challenged with the dopamine precursor L-DOPA they turn towards the unlesioned side. This pattern of rotation can also be elicited by dopaminergic agonists and it is likely due to post-synaptic supersensitivity after lesioning the ipsilateral dopaminergic input to the striatum. Those animals in which L-DOPA induced a strong rotational behaviour expressed high levels of *c-fos* in the ipsilateral striatum, nucleus accumbens, and areas of neocortex; no increase in FLIR was seen in areas contralateral to the lesion. This suggests that the response was secondary to dopaminergic post-synaptic supersensitivity (Robertson *et al.*, 1989a). A similar pattern of *c-fos* activation and rotational behaviour was elicited by D1-selective agonists (Robertson *et al.*, 1989b). Amphetamine, which induces the release of endogenous dopamine, elicits ipsilateral rotational behaviour by acting through the undamaged striatum. Similarly, *c-fos* activation after administration of amphetamine would occur in the striatum contralateral to a 6-OHDA lesion of the SN (Robertson *et al.*, 1989b). However, rotational behaviour and *c-fos* expression could be dissociated.

Lesioning of the SN with 6OHDA had no long-term effect on *c-fos* expression; however, there was a large widespread and long-lasting increase in FRAs expression in the striatum ipsilateral to the side of the lesion (Dragunow *et al.*, 1991b).

Administration of a selective D2 dopamine agonist (LY 171555) in SN-lesioned animals resulted in contraversive rotation but no *c-fos* activation was seen (Robertson *et al.*, 1989b). Furthermore, administration of a D1 agonist induced *c-fos* in the ipsilateral striatum, even if the animals were under anaesthesia and rotational behaviour was prevented. Direct injection of a D1 agonist into SN induced rotational behaviour but not activation of *c-fos*

(Robertson *et al.*, 1989b). Cocaine and amphetamine (after a single systemic dose) both induced *c-fos* in the caudate-putamen (Graybiel *et al.*, 1990; Young *et al.*, 1991), but in a different pattern (Graybiel *et al.*, 1990). The effects of both drugs were blocked, to a great extent, by selective D1 antagonists but not by D2 antagonists, which by themselves can induce *c-fos* (see below). The pattern of *c-fos* activation induced by amphetamine was most pronounced in the striosomal compartment of the striatum. Cocaine-induced *c-fos* activation was seen in both the striosomal and matrix compartments (Graybiel *et al.*, 1990).

As mentioned above, unilateral lesion of the SN or its efferents to the striatum results in a model of unilateral Parkinson's disease, in which the rats become supersensitive to dopaminergic agonists. The D1, but not D2, agonists in such treated animals will induce *c-fos*. After SN lesioning, a D1 agonist produced *c-fos* activation in striatal neurons (retrogradely labelled) projecting to the SNr. These findings suggest that D1 agonists activate *c-fos* in the medium-sized neurons which project to the SNr (Robertson *et al.*, 1992).

Interestingly, D1 agonists induce *c-fos* in SN-lesioned animals but not in naive rats. Though D2 agonists seem to have no effect on *c-fos* expression, the combination of D1 and D2 agonists (on lesioned animals) had synergistic effect on the induction of not only *c-fos* but also in the IEG *c-jun*, *jun-B*, and *NGF-1A*. The administration of a low dose D1 agonist (SKF-38393) only results in a diffuse, low level, expression of *c-fos*. Administration of a low dose of a D2 agonist (quinpirole) results in little contraversive rotation and no *c-fos* induction in the caudate-putamen but produces some *c-fos* induction in the globus pallidus (Robertson *et al.*, 1992; Paul *et al.*, 1992). The combination of D1 and D2 agonists at low doses resulted in a synergistic effect in rotational behaviour, as previously described (Robertson and Robertson, 1986, 1989) and in a compartmentalized pattern of *c-fos* activation in the striatum (Paul *et al.*, 1992). The FLIR was concentrated in striosomes and dorsolateral caudate-putamen; Northern blot analysis showed *c-fos* mRNA expression only after combination of dopaminergic agonists. The FLIR was blocked with pre-administration of D1- and D2-selective antagonists. Interestingly, *c-fos* expression was also reduced if prior to the combination of D1 and D2 agonists the animals received MK-801, a NMDA receptor antagonist (Paul *et al.*, 1992).

Administration of D2 antagonists such as haloperidol, which is also a sigma receptor antagonist, induced *c-Fos* protein and FRA in striatal neurons (Dragunow *et al.*, 1990d; Miller, 1990). This effect was inhibited by the NMDA receptor antagonist MK-801 (Dragunow *et al.*, 1990b). It was hypothesized that activation of *c-fos* was secondary to blockade of inhibitory presynaptic dopaminergic receptors in the glutamatergic corticostriatal pathway, thus inducing the release of glutamate which in turn would activate *c-fos* through an NMDA receptor mediated mechanism. More evidence for this potential mechanism came from a study which showed that electrical stimulation of a broad area of fronto-parietal cortex induced *c-fos* in the striatum

(Aronin *et al.*, 1991; Fu and Beckstead, 1992). Lesioning of the SN had no effect on the induction of *c-fos* in the striatum. Another study investigating the effect of haloperidol on the expression of *c-fos* mRNA showed that the induction of *c-fos* in the striatum was dose dependent. Furthermore, a D2 agonist completely reversed the *c-fos* induction (Miller, 1990).

Recent studies demonstrated that administration of an antisense oligonucleotide (AON) to *c-fos* mRNA blocks the *in vivo* expression of *c-fos* IR (Chiasson *et al.*, 1992; Dragunow *et al.*, 1993; Sommer *et al.*, 1993; Hooper *et al.*, 1994; Liu *et al.*, 1994). Furthermore, Sommer *et al.* (1993) and Dragunow *et al.* (1993) demonstrated that infusion of AON to *c-fos* mRNA into the striatum induced ipsilateral rotational behaviour after D-amphetamine and apomorphine administration. This effect was not produced by sense ON or unrelated ON, indicating a role for *c-fos* in control of behavioural activity.

4.7.2. Activation of *c-fos* Mediated by Adrenergic Receptors

The role of adrenergic receptors in the activation of *c-fos* was investigated with specific adrenergic agonists and antagonists (Gubits *et al.*, 1989; Bing *et al.*, 1991, 1992; Stone *et al.*, 1991, 1992). Administration of yohimbine, an α_2 adrenoreceptor antagonist produces an increase in total *c-fos* mRNA in the brain and CFLIR, an effect that is partially inhibited, independently, by the β adrenoreceptor antagonist propranolol, by the α_2 agonist clonidine and the α_1 antagonist prazosin (Gubits *et al.*, 1989; Bing *et al.*, 1992). Increase in CFLIR following the administration of yohimbine was observed in the locus ceruleus, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the nucleus of the tractus solitarius, ventrolateral medulla oblongata, and the paraventricular nucleus (Tsujino *et al.*, 1992). Clonidine induced CFLIR in the oxytocinergic neurons in the paraventricular and supraoptic nuclei in the hypothalamus (Tsujino *et al.*, 1992). Thus, it appears that adrenoreceptors, especially α_2 , are involved in the regulation of *c-fos* expression in neurons related mainly with autonomic functions.

4.7.3. Other Pharmacological Agents

Administration of morphine induced *c-fos* mRNA and CFLIR in the caudate-putamen (Chang *et al.*, 1988), an effect that was inhibited by the m-type opiate receptor antagonist naloxone. Maximum of *c-fos* mRNA levels were achieved within 45 min after the injection of morphine and was back to basal levels after 90 min.

Cholinergic agonists can be divided into nicotinic and muscarinic and their role in the regulation of *c-fos* is well documented (Pang *et al.*, 1993; Bernard *et al.*, 1993; Gudehithlu *et al.*, 1993). Acute injections of nicotine induces CFLIR in neurons in the medial terminal nucleus of the accessory optic system, the superior colliculus, the interpeduncular nucleus and in the caudal linear subnucleus of the ventral tegmental area, mostly in non-dopaminergic neurons (Pang *et al.*, 1993). Administration of the muscarinic

agonist pilocarpine induced *c-fos* expression in the cortex, an effect that was potentiated by lithium and inhibited by the m1 muscarinic antagonist pirenzepine (Weiner *et al.*, 1991).

A role for serotonin in the activation of *c-fos* has been reported (Richard *et al.*, 1992; Bhat and Baraban, 1993; Pretel and Piekut, 1991; Leslie *et al.*, 1993a, 1993b). The serotonergic agonist fenfluramine produced an increase in CFLIR in the caudate-putamen, the paraventricular nucleus of the hypothalamus and in the central amygdaloid nucleus (Richard *et al.*, 1992).

The levels of *c-fos* mRNA were increased in the caudate putamen after administration of caffeine, an effect that was blocked by the A2 adenosinergic antagonist NECA (Nakajima *et al.*, 1988, 1989).

In summary, multiple neurotransmitters systems are involved in the regulation of *c-fos* expression in the brain.

4.8. Somatosensory and Nociceptive Stimulation of *c-fos* Expression

Noxious stimulation can induce *c-fos* activation in the dorsal horn of the spinal cord (Hunt *et al.*, 1987). Cutaneous application of noxious substance to the hind limb induced FLIR in neurons, mostly located in layers I and II, but also in layers V, VI, VII and X. Following non-noxious brushing and manipulation in the hind limb labelled nuclei were seen in layers II-IV and rarely in layer I. No *c-fos* was seen in the dorsal root ganglion, nucleus gracilis or ventral horn following noxious chemical or radiant heat stimulation (Hunt *et al.*, 1987). Most of the labelled neurons, after noxious somatic stimulation, were found in lamina I, the outer part of laminae II, V and VI and to a lesser extent in laminae VII, VIII and X. All of the labelled neurons were located ipsilateral to the injured hindpaw (Menetrey *et al.*, 1989) except for bilateral labelling in lamina VIII. The rostrocaudal spread in CFLIR was more extensive in deeper laminae (Menetrey *et al.*, 1989). Similar results were reported by several groups (Draisci and Iadarola, 1989). Rostrocaudal spread of CFLIR was more circumscribed to fewer segments of the SC if the stimuli was injection of formalin to a paw rather than saline (Abbadie *et al.*, 1994; Abbadie and Besson, 1992; Gogas *et al.*, 1993). The pattern of CFLIR after visceral stimulation was bilateral, with a much more rostrocaudal spread particularly in the superficial dorsal horn (Menetrey *et al.*, 1989). Furthermore, after noxious stimulation, retrograde labelling demonstrated projection neurons (to brainstem and thalamus) of lamina I, lateral neck of the dorsal horn and laminae VIII and X with FLI. Most CFLIR was found in neurons within the medial two-thirds of the superficial laminae (Bullitt *et al.*, 1992; Noguchi *et al.*, 1992). Stronger stimulation produced increased expression of FLIR in neurons located at deeper layers (Bullitt *et al.*, 1992).

Further studies have shown a differential pattern of *c-fos* expression in the spinal cord in mechanically versus chemically induced visceral nociception (De Leo *et al.*, 1991). Intraperitoneal injection of acetic acid induced FLIR bilaterally in laminae I and X predominantly in the thoracolumbar area; in

contrast, distention of the duodenum with a balloon led to a greater number, and more intense labelling in laminae I-VI, IX and X. These findings support the idea that these two types of nociceptive information are processed differently (De Leo *et al.*, 1991).

A stimulus-induced sensitization appears to occur after presentation of a noxious stimulus (Leah *et al.*, 1992). Thus, a larger number of neurons show FLIR after a noxious stimulus if it was preceded by a similar stimulus within 1-2 hr (Leah *et al.*, 1992).

Chemical noxious stimulation to the nasal mucosa resulted in FLIR in the brainstem; specifically in laminae I and II of the subnucleus caudalis; in the nucleus interpolaris of the trigeminal brainstem nuclear complex; and, in the medullary lateral reticular nucleus (Anton *et al.*, 1991).

Though noxious stimuli can induce FLIR in the spinal cord, sensory, non-nociceptive, stimulation, as initially shown by Hunt *et al.* (1987), was enough to induce CFLIR. Tactile stimuli can induce several transcription factors in the somatosensory cortex (Mack and Mack, 1992). Tactile stimuli delivered with a paint brush stroking a rat's whiskers unilaterally for a 15 min period and Krox-24 (NGF-IA), NGF-IB and CFLIR were compared in the cortices ipsilateral and contralateral to the stimulation. The induction of IR was predominant in layer IV of somatosensory cortex contralateral to the stimulus (Mack and Mack, 1992).

Double immunolabelling studies have shown that most neurons in superficial laminae of the dorsal horn expressing FLIR after noxious stimulation received encephalinerig, serotoninerig and substance P inputs (Pretel and Piekut, 1991). In addition, recent studies have focused on the role of nitric oxide (NO) on *c-fos* expression after noxious stimulation. Lee *et al.* (1992), showed that intrathecal administration of a NO synthetase (NOS) inhibitor significantly reduced the number of cells with FLIR in the dorsal horn after noxious stimulation of the hind paw. Furthermore, there was co-expression of FLIR and NOS IR in some spinothalamic neurons after noxious stimulation (Lee *et al.*, 1993).

5. ACTIVATION OF *c-fos* DURING DEVELOPMENT

This section discusses the expression of *c-fos* during brain development both under basal conditions and after specific stimuli.

5.1. Basal Levels

Gubits *et al.* (1988) studied the levels of *c-fos* expression in the brain at different stages of development. They showed that higher levels of *c-fos* mRNA occurred in the rat brain between birth and postnatal day 16 (P16). The highest levels of *c-fos* mRNA were seen in meninges at embryonic day 18 (E18) and cerebral cortex and cerebellum at P30. At this stage (P30), only low levels of *c-fos* mRNA were detected in the hippocampus and in striatum. The levels of *c-fos* mRNA in the whole brain attained at P30 were maintained up to 1.5 years of age in male

rats. A transient decrease in these levels was seen at P50, raising the question of whether this decrease is related to the onset of puberty in males. It was suggested that the expression of *c-fos* could be related to synaptogenesis and differentiation rather than to mitosis. An *in situ* hybridization study by Caubert (1989) investigated the expression of *c-fos* mRNA in the developing brain. Around embryonic day 12 (E12), high levels of *c-fos* were seen throughout the CNS. At later stages, the expression of *c-fos* was confined to limited areas of the cerebellum, spinal cord, forebrain and retina (Caubert, 1989).

A *fos-lacZ* transgenic mouse had been recently used to study the developmental expression of the transgene (Smeyne *et al.*, 1993a). Expression of the transgene that was observed at P0 in olfactory bulb, hippocampus, retrosplenial cortex and thalamic nuclei, decreased to adult levels by 3 weeks. This, again, suggests that *c-fos* may play a role in the CNS during development (Smeyne *et al.*, 1992a). Northern blot analysis has shown low levels of *c-fos* mRNA which increases within P15-P30 (Gubits *et al.*, 1988; McCormack *et al.*, 1992).

5.2. Seizures

Developmental differences in expression of *c-fos* in the brain have also been observed after seizure induction (Schreiber *et al.*, 1992; Jensen *et al.*, 1993). The PTZ administration in rats resulted in increase in *c-fos* immunoreactivity in superficial layers of retrosplenial, cingulate and neocortex in adults but only deep layers in P10 rats were affected (Jensen *et al.*, 1993). Similarly, KA administration resulted in increase in *c-fos* expression in limbic structures in adult animals but not in pups prior to P13 (Schreiber *et al.*, 1992).

The effects of PTZ, fluoroethyl and hypoxia were studied in immature rats (postnatal day 10) and compared with that in adult rats. The CFLIR after PTZ or fluoroethyl-induced seizure was elevated in amygdala, piriform cortex and hypothalamus at both ages; superficial layers of retrosplenial, cingulate and neocortex were stained in adults but staining was confined to deep layers of neocortex in P10 rats. Intense staining at 2 hr after seizure onset was reduced at 4 hr in adults but IR was not seen until 4-6 hr after seizure in P10 rats. The CFLIR within the dentate gyrus and hippocampus occurred only after prolonged seizures. Hypoxia, which produced seizures in P10 rats, induced CFLIR in the layer VI of neocortex but rarely involved limbic structures (Jensen *et al.*, 1993).

5.3. Brain Injury

Differential regulation of *c-fos* expression during development after brain injury has been reported (Herrera *et al.*, 1993a). Rats that were inflicted with a mechanical cortical injury at post-natal day (PD) 10 or 15, and sacrificed 1.5 hr after, did not show an increase in *c-fos* immunoreactivity far from the wound, but some of these animals did show *c-fos* immunoreactive (IR) nuclei close to the lesion (PD 15). At PD 22, cortical injury produced an increase in *c-fos* IR nuclei in the piriform cortex ipsilateral but

not contralateral to the lesion. This pattern was maintained up to PD 360 (Herrera *et al.*, 1993a). Similarly, the presence of *c-fos* IR cells was observed in the ipsilateral cingulate cortex from PD 22 onward.

Ruppert and Wille (1987) showed that disruption of the cerebellum at P3 and up to P7 was followed by a massive increase in *c-fos* mRNA. This effect was not seen in the adult animal with similar injury. These findings show a clear differential regulation of *c-fos* during development and may help to explain, at least partially, the different clinical outcomes seen in children and in adults after head trauma.

6. CONCLUSIONS

Activation of *c-fos* in the brain can be induced by a diverse group of stimuli. The inducibility of *c-fos* has been used as a tool to study neuronal activation in different systems in the brain and now *c-fos* can be regarded as one of several IEGs that may link external cellular signals with phenotypic changes in brain cells. Different sets of IEGs may be activated by different stimuli and, in turn, these IEGs may induce or inhibit the transcription of late or target genes which may, in turn, produce long-term changes. The exact role of *c-fos* in the brain is not clear yet; however, there is direct evidence of a role for Fos in the control of behavioural activity (Sommer *et al.*, 1993). Even if the exact role of *c-fos* in the brain has not yet been elucidated, its detection has led to a further understanding of different systems in the CNS and allows the mapping of neuronal populations activated by a variety of external stimuli.

Acknowledgements—The authors thank Drs D. Hewitt, N. Schiff, B. Volpe for reading this manuscript and giving their helpful comments.

REFERENCES

- Abbadie, C., Honore, P. and Besson, J. M. (1994) Intense cold noxious stimulation of the rat hindpaw induces *c-fos* expression in lumbar spinal cord neurons. *Neuroscience* 59, 457-468.
- Abbadie, C. and Besson, J. M. (1992) *C-fos* expression in rat lumbar spinal cord during the development of adjuvant-induced arthritis. *Neuroscience* 48, 985-993.
- Abe, H. and Rusak, B. (1992) Stimulation of the hamster ventral lateral geniculate nucleus induces Fos-like immunoreactivity in suprachiasmatic nucleus cells. *Neurosci. Lett.* 148, 185-189.
- Abe, H., Rusak, B. and Robertson, H. A. (1991) Photic induction of Fos protein in the suprachiasmatic nucleus is inhibited by the NMDA receptor antagonist MK-801. *Neurosci. Lett.* 127, 9-12.
- Abe, H., Rusak, B. and Robertson, H. A. (1992) NMDA and non-NMDA receptor antagonists inhibit photic induction of Fos protein in the hamster suprachiasmatic nucleus. *Brain Res. Bull.* 28, 831-835.
- Abraham, W. C., Dragunow, M. and Tate, W. P. (1992) The role of immediate early genes in the stabilization of long-term potentiation. *Mol. Neurobiol.* 5, 297-314.
- Alcantara, A. A. and Greenough, W. T. (1993) Developmental regulation of *fos* and *fos*-related antigens in cerebral cortex, striatum, hippocampus, and cerebellum of the rat. *J. Comp. Neurol.* 334, 75-85.
- Amir, S., Robinson, B. and Woodside, B. (1994) Induction of Fos protein in the piriform cortex after brain injury in pentobarbital-anesthetized rats; lack of effect of lactation. *Brain Res.* 652, 341-345.
- An, G., Lin, T. N., Liu, J. S., Xue, J. J., He, Y. Y. and Hsu, C. Y.

- (1993) Expression of *c-fos* and *c-jun* family genes after focal cerebral ischemia [see comments]. *Ann. Neurol.* 33, 457-464.
- Anderson, A. J., Cummings, B. J. and Cotman, C. W. (1994) Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. *Expl. Neurol.* 125, 286-295.
- Anokhin, K. V., Mileusnic, R., Shamakina, I. Y. and Rose, S. P. (1991) Effects of early experience on *c-fos* gene expression in the chick forebrain. *Brain Res.* 544, 101-107.
- Anton, F., Herdegen, T., Peppel, P. and Leah, J. D. (1991) *C-fos*-like immunoreactivity in rat brainstem neurons following noxious chemical stimulation of the nasal mucosa. *Neuroscience* 41, 629-641.
- Arenander, A. T., deVellis, J. and Herschman, H. R. (1989a) Induction of *c-fos* and TIS genes in cultured rat astrocytes by neurotransmitters. *J. Neurosci. Res.* 24, 107-114.
- Arenander, A. T., Lim, R. W., Varnum, B. C., Cole, R., De Vellis, J. and Herschman, H. R. (1989b) TIS gene expression in cultured rat astrocytes: induction by mitogens and stellation agents. *J. Neurosci. Res.* 23, 257-265.
- Aronin, N., Chase, K., Sagar, S. M., Sharp, F. R. and Di Figlia, M. (1991) N-methyl-D-aspartate receptor activation in the neostriatum increases *c-fos* and *fos*-related antigens selectively in medium-sized neurons. *Neuroscience* 44, 409-420.
- Arnold, F. J., De-Lucas-Bueno, M., Shiers, H., Hancock, D. C., Evan, G. I. and Herbert, H. (1992) Expression of *c-fos* in regions of the basal limbic forebrain following intracerebroventricular corticotropin-releasing factor in unstressed or stressed male rats. *Neuroscience* 51, 377-390.
- Bading, H., Ginty, D. D. and Greenberg, M. E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* 260, 181-186.
- Bele, S., Gass, P., Herdegen, T., Bravo, R. and Kiessling, M. (1994) Concurrent immediate early gene induction by epileptic seizures in heterotopic cortical grafts and neocortex. *Brain Res. Mol. Brain Res.* 22, 9-19.
- Bernard, V., Dumartin, B., Lamy, E. and Bloch, B. (1993) Fos immunoreactivity after stimulation or inhibition of muscarinic receptors indicates anatomical specificity for cholinergic control of striatal efferent neurons and cortical neurons in the rat. *Eur. J. Neurosci.* 5, 1218-1225.
- Bhat, R. V. and Baraban, J. M. (1993) Activation of transcription factor genes in striatum by cocaine: role of both serotonin and dopamine systems. *J. Pharmacol. Exp. Ther.* 267, 496-505.
- Bing, G., Stone, E. A., Zhang, Y. and Filer, D. (1992) Immunohistochemical studies of noradrenergic-induced expression of *c-fos* in the rat CNS. *Brain Res.* 592, 57-62.
- Bing, G. Y., Filer, D., Miller, J. C. and Stone, E. A. (1991) Noradrenergic activation of immediate early genes in rat cerebral cortex. *Brain Res. Mol. Brain Res.* 11, 43-46.
- Bliss, T. V. P. and Lomo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol. (Lond.)* 232, 331-356.
- Blumenfeld, K. S., Welsh, F. A., Harris, V. A. and Pesenson, M. A. (1992) Regional expression of *c-fos* and heat shock protein-70 mRNA following hypoxia-ischemia in immature rat brain. *J. Cereb. Blood Flow Metab.* 12, 987-995.
- Bokesch, P. M., Marchand, J. E., Connelly, C. S., Wurm, W. H. and Kream, R. M. (1994) Dextrometorphan inhibits ischemia-induced *c-fos* expression and delayed neuronal death in hippocampal neurons. *Anesthesiology* 81, 470-477.
- Brown, I. R. (1990) Induction of heat shock (stress) genes in the mammalian brain by hyperthermia and other traumatic events: a current perspective. *J. Neurosci. Res.* 27, 247-255.
- Bullitt, E., Lee, C. L., Light, A. R. and Willcockson, H. (1992) The effect of stimulus duration on noxious-stimulus induced *c-fos* expression in the rodent spinal cord. *Brain Res.* 580, 172-179.
- Campeau, S., Hayward, M. D., Hope, B. T., Rosen, J. B., Nestler, E. J. and Davis, M. (1991) Induction of the *c-fos* proto-oncogene in rat amygdala during unconditioned and conditioned fear. *Brain Res.* 565, 349-352.
- Carter, D. A. (1993) Differential intracellular mechanisms mediate the co-ordinate induction of *c-fos* and Jun-B in the rat pineal gland. *Eur. J. Pharmacol.-Mol. Pharmacol. Sect.* 244, 285-291.
- Caubert, J. (1989) *C-fos* proto-oncogene expression in the nervous system during mouse development. *Mol. Cell Biol.* 9, 2269-2272.
- Ceccatelli, S., Villar, M. J., Goldstein, M. and Hokfelt, T. (1989) Expression of *c-fos* immunoreactivity in transmitter-characterized neurons after stress. *Proc. natn. Acad. Sci. U.S.A.* 86, 9569-9573.
- Chang, S. L., Squinto, S. P. and Harlan, R. E. (1988) Morphine activation of *c-fos* expression in rat brain. *Biochem. Biophys. Res. Commun.* 157, 698-704.
- Chastrette, N., Pfaff, D. W. and Gibbs, R. B. (1991) Effects of daytime and nighttime stress on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus, the habenula, and the posterior paraventricular nucleus of the thalamus. *Brain Res.* 563, 339-344.
- Chen, S. and Hillman, D. E. (1992) Transient *c-fos* expression and dendritic spine plasticity in hippocampal granule cells. *Brain Res.* 577, 169-174.
- Chiasson, B. J., Hooper, M. L., Murphy, P. R. and Robertson, H. A. (1992) Antisense oligonucleotide eliminates *in vivo* expression of *c-fos* in mammalian brain. *Eur. J. Pharmacol.* 227, 451-453.
- Christensen, T., Jorgensen, M. B. and Diemer, N. H. (1993) Impairment of Fos protein formation in the rat infarct border zone by MK-801, but not by NBQX. *Acta Neurol. Scand.* 87, 510-515.
- Clark, M., Post, R. M., Weiss, S. R., Cain, C. J. and Nakajima, T. (1991) Regional expression of *c-fos* mRNA in rat brain during the evolution of amygdala kindled seizures. *Brain Res. Mol. Brain Res.* 11, 55-64.
- Cohen, D. R. and Curran, T. (1990) Analysis of dimerization and DNA binding functions in Fos and Jun by domain-swapping: involvement of residues outside the leucine zipper/basic region. *Oncogene* 5, 929-939.
- Cole, A. J., Saffen, D. W., Baraban, J. and Worley, P. F. (1989) Rapid increase of an immediate-early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* 340, 474-476.
- Colwell, C. S. and Foster, R. G. (1992) Photic regulation of Fos-like immunoreactivity in the suprachiasmatic nucleus of the mouse. *J. Comp. Neurol.* 324, 135-142.
- Colwell, C. S., Kaufman, C. M. and Menaker, M. (1993) Photic induction of Fos in the hamster suprachiasmatic nucleus is inhibited by baclofen but not by diazepam or bicucullin. *Neurosci. Lett.* 163, 177-181.
- Combs, D. J., Dempsey, R. J., Donaldson, D. and Kindy, M. S. (1992) Hyperglycemia suppresses *c-fos* mRNA expression following transient cerebral ischemia in gerbils. *J. Cereb. Blood Flow Metab.* 12, 169-172.
- Condorelli, D. F., Kaczmarek, L., Nicoletti, F., Arcidiacono, A., Dell'Albani, P., Ingrassia, F., Magri, G., Malaguarnera, L., Avola, R. and Messina, A. (1989) Induction of proto-oncogene *fos* by extracellular signals in primary glial cell cultures. *J. Neurosci. Res.* 23, 234-239.
- Covenas, R., de Leon, M., Cintra, A., Bjelke, B., Gustafsson, J. A. and Fuxe, K. (1993) Coexistence of *c-fos* and glucocorticoid receptor immunoreactivities in the CRF immunoreactive neurons of the paraventricular hypothalamic nucleus of the rat after acute immobilization stress. *Neurosci. Lett.* 149, 149-152.
- Curran, T. (1988) The *fos* oncogene. In: *The Oncogene Handbook*, pp. 307-325. Eds. E.P. Reddy and A.M. Skalka. Elsevier: Amsterdam.
- Curran, T., Miller, A. D., Zokas, L. and Verma, I. M. (1984) Viral and cellular *fos* proteins, a comparative analysis. *Cell* 36, 259-268.
- Curran, T., Van Beveren, C., Ling, N. and Verma, I. M. (1985) Viral and cellular *fos* proteins are complexed with a 39,000 dalton cellular protein. *Mol. Cell Biol.* 5, 167-172.
- Curran, T. and Morgan, J. I. (1985) Superinduction of *c-fos* by nerve growth factor in the presence of peripherally active benzodiazepines. *Science* 229, 1265-1268.
- Curran, T. and Teich, N. M. (1982) Candidate product of the FBJ murine osteosarcoma virus oncogene: characterization of a 55,000 dalton phosphoprotein. *J. Virol.* 42, 114-122.
- Dave, J. R., Tabakoff, B. and Hoffman, P. L. (1990) Ethanol withdrawal seizures produce increased *c-fos* mRNA in mouse brain. *Mol. Pharmacol.* 37, 367-371.
- De Leo, J. A., Coombs, D. W. and McCarthy, L. E. (1991) Differential c-Fos-like protein expression in mechanically versus chemically induced visceral nociception. *Brain Res. Mol. Brain Res.* 11, 167-170.
- Didier, M., Roux, P., Piechaczyk, M., Verrier, B., Bockaert, J. and Pin, J. P. (1989) Cerebellar granule cell survival and maturation

- induced by K^+ and NMDA correlate with *c-fos* proto-oncogene expression. *Neurosci. Lett.* 107, 55–62.
- Ding, J. M., Carver, W. C., Terracio, L. and Buggy, J. (1994) Proto-oncogene *c-fos* and the regulation of vasopressin gene expression during dehydration. *Brain Res. Mol. Brain Res.* 21, 247–255.
- Dony, C. and Gruss, P. (1987) Proto-oncogene *c-fos* expression in growth regions of fetal bone and mesodermal web tissue. *Nature* 328, 711–714.
- Douglas, R., Dragunow, M. and Robertson, H. A. (1988) High frequency discharge of dentate granule cells, but not long-term potentiation, induces *c-fos* protein. *Mol. Brain Res.* 4, 259–262.
- Dragunow, M., Peterson, M. R. and Robertson, H. A. (1987) Presence of *c-fos* like immunoreactivity in the adult rat brain. *Eur. J. Pharmacol.* 135, 113–114.
- Dragunow, M., Robertson, H. A. and Robertson, G. S. (1988) Amygdala kindling and *c-Fos* protein(s). *Expl Neurol.* 102, 261–263.
- Dragunow, M., Abraham, W. C., Goulding, M., Mason, S. E., Robertson, H. A. and Faull, R. L. M. (1989a) Long-term potentiation and the induction of *c-fos* mRNA and proteins in the dentate gyrus of unanesthetized rats. *Neurosci. Lett.* 101, 274–280.
- Dragunow, M., Currie, R. W., Robertson, H. A. and Faull, R. L. (1989b) Heat shock induces *c-Fos* protein-like immunoreactivity in glial cells in adult rat brain. *Expl Neurol.* 106, 105–109.
- Dragunow, M., de Castro, D. and Faull, R. L. M. (1990a) Induction of Fos in glia-like cells after local brain injury but not during Wallerian degeneration. *Brain Res.* 527, 41–54.
- Dragunow, M., Faull, R. L. and Jansen, K. L. (1990b) MK-801, an antagonist of NMDA receptors, inhibits injury-induced *c-Fos* protein accumulation in rat brain. *Neurosci. Lett.* 109, 128–133.
- Dragunow, M., Goulding, M., Faull, R. L., Ralph, R., Mee, E. and Friih, R. (1990c) Induction of *c-fos* mRNA and protein in neurons and glia after traumatic brain injury: pharmacological characterization. *Expl Neurol.* 107, 236–248.
- Dragunow, M., Robertson, G. S., Faull, R. L., Robertson, H. A. and Jansen, K. (1990d) D2 dopamine receptor antagonists induce Fos and related proteins in rat striatal neurons. *Neuroscience* 37, 287–294.
- Dragunow, M., Williams, M. and Faull, R. L. (1990e) Haloperidol induces Fos and related molecules in intrastriatal grafts derived from fetal striatal primordia. *Brain Res.* 530, 309–311.
- Dragunow, M., Faull, R. L., Waldvogel, H. J., Williams, M. N. and Leah, J. (1991a) Elevated expression of Jun- and Fos-related proteins in transplanted striatal neurons. *Brain Res.* 558, 321–324.
- Dragunow, M., Leah, J. D. and Faull, R. L. (1991b) Prolonged and selective induction of Fos-related antigen(s) in striatal neurons after 6-hydroxydopamine lesions of the rat substantia nigra pars compacta. *Brain Res. Mol. Brain Res.* 10, 355–358.
- Dragunow, M. (1992) Axotomized medial septal-diagonal band neurons express Jun-like immunoreactivity. *Brain Res. Mol. Brain Res.* 15, 141–144.
- Dragunow, M., Lawlor, P., Chiasson, B. and Robertson, H. (1993) *C-fos* antisense generates apomorphine and amphetamine-induced rotation. *Neuroreport* 5, 305–306.
- Dragunow, M. and Faull, R. (1989) The use of *c-fos* as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Meth.* 29, 261–265.
- Dragunow, M. and Faull, R. L. (1990) MK-801 induces *c-Fos* protein in thalamic and neocortical neurons of rat brain. *Neurosci. Lett.* 113, 144–150.
- Dragunow, M. and Robertson, H. A. (1987a) Kindling stimulation induces *c-Fos* protein(s) in granule cells of the rat dentate gyrus. *Nature* 329, 441–442.
- Dragunow, M. and Robertson, H. A. (1987b) Generalized seizures induce *c-Fos* protein(s) in mammalian neurons. *Neurosci. Lett.* 82, 157–161.
- Dragunow, M. and Robertson, H. A. (1988a) Localization and induction of *c-Fos* protein-like immunoreactive material in the nuclei of adult mammalian neurons. *Brain Res.* 440, 252–260.
- Dragunow, M. and Robertson, H. A. (1988b) Brain injury induces *c-Fos* protein(s) in nerve and glial-like cells in adult mammalian brain. *Brain Res.* 455, 295–299.
- Draisci, G. and Iadarola, M. J. (1989) Temporal analysis of increases in *c-fos*, preprodynorphin and preproenkephalin mRNAs in rat spinal cord. *Brain Res. Mol. Brain Res.* 6, 31–37.
- D'Costa, A., Breese, C. R., Boyd, R. L., Booz, R. M. and Sonntag, W. E. (1991) Attenuation of *fos*-like immunoreactivity induced by a single electroconvulsive shock in brains of aging mice. *Brain Res.* 567, 204–211.
- Earnest, D. J., Iadarola, M., Yeh, H. H. and Olschowka, J. A. (1990) Photoc regulation of *c-fos* expression in neural components governing the entrainment of circadian rhythms. *Expl Neurol.* 109, 353–361.
- Earnest, D. J., Ouyang, S. and Olschowka, J. A. (1992) Rhythmic expression of Fos-related proteins within the rat suprachiasmatic nucleus during constant retinal illumination. *Neurosci. Lett.* 140, 19–24.
- Earnest, D. J., Di Giorgio, S. and Olschowka, J. A. (1993) Light induces expression of Fos-related proteins within gastrin-releasing peptide neurons in the rat suprachiasmatic nucleus. *Brain Res.* 627, 205–209.
- Franza, B. R. Jr., Sambucetti, L. C., Cohen, D. R. and Curran, T. (1987) Analysis of Fos protein complexes and Fos-related antigens by high resolution two-dimensional gel electrophoresis. *Oncogene* 1, 213–221.
- Franza, B. R. Jr., Rauscher, F. J. III, Josephs, S. F. and Curran, T. (1988) The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 sites. *Science* 239, 1150–1153.
- Fu, L. and Beckstead, R. M. (1992) Cortical stimulation induces Fos expression in striatal neurons. *Neuroscience* 46, 329–334.
- Gass, P., Spranger, M., Herdegen, T., Bravo, R., Kock, P., Hacke, W. and Kiessling, M. (1992) Induction of Fos and Jun proteins after focal ischemia in the rat: differential effect of the N-methyl-D-aspartate receptor antagonist MK-801. *Acta Neuropathol. (Berl.)* 84, 545–553.
- Ghosh, A., Ginty, D. D., Bading, H. and Greenberg, M. E. (1994) Calcium regulation of gene expression in neuronal cells. *J. Neurobiol.* 25, 294–303.
- Giovannelli, L., Shiromani, P. J., Jirkowski, G. F. and Bloom, F. E. (1990) Oxytocin neurons in the rat hypothalamus exhibit *c-fos* immunoreactivity upon osmotic stress. *Brain Res.* 531, 299–303.
- Giovannelli, L., Shiromani, P. J., Jirkowski, G. F. and Bloom, F. E. (1992) Expression of *c-Fos* protein by immunohistochemically identified oxytocin neurons in the rat hypothalamus upon osmotic stimulation. *Brain Res.* 588, 41–48.
- Goddard, G. V., McIntyre, D. C. and Leech, C. K. (1969) A permanent change in brain function resulting from daily electrical stimulation. *Expl Neurol.* 25, 295–330.
- Goebel, P., Castelucci, V. F., Schaefer, S. and Kandel, E. R. (1986) The long and the short of long-term memory — a molecular framework. *Nature* 322, 419–422.
- Gogas, K. R., Levine, J. D. and Basbaum, A. I. (1993) Mechanisms of opioid-mediated antinociception: correlation of Fos expression and behavior. *NIDA Research Monograph* 125, 134–160.
- Graybiel, A. M., Moratalla, R. and Robertson, H. A. (1990) Amphetamine and cocaine induce drug-specific activation of the *c-fos* gene in striosome-matrix compartments and limbic subdivisions of the striatum. *Proc. natn. Acad. Sci. U.S.A.* 87, 6912–6916.
- Greenberg, M. E., Ziff, E. B. and Greene, L. A. (1986) Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science* 234, 80–83.
- Greenberg, M. E. and Ziff, E. B. (1984) Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature* 311, 433–438.
- Gubits, R. M., Hazelton, J. L. and Simantov, R. (1988) Variations in *c-fos* gene expression during rat brain development. *Mol. Brain Res.* 3, 197–202.
- Gubits, R. M., Smith, T. M., Fairhurst, J. L. and Yu, H. (1989) Adrenergic receptors mediate changes in *c-fos* mRNA levels in brain. *Brain Res. Mol. Brain Res.* 6, 39–45.
- Gudeithlu, K. P., Neff, N. H. and Hadjiconstantinou, M. (1993) *C-fos* and NGFI-A mRNA of rat retina: evidence for light-induced augmentation and a role for cholinergic and glutamate receptors. *Brain Res.* 631, 77–82.
- Gunn, A. J., Dragunow, M., Faull, R. L. and Gluckman, P. D. (1990) Effects of hypoxia-ischemia and seizures on neuronal and glial-like *c-fos* protein levels in the infant rat. *Brain Res.* 531, 105–116.
- Harbuz, M. S., Chalmers, J., De-Souza, L. and Lightman, S. L. (1993) Stress-induced activation of CRF and *c-fos* mRNAs in the paraventricular nucleus are not affected by serotonin depletion. *Brain Res.* 609, 167–173.

- Hayward, M. D., Duman, R. S. and Nestler, E. J. (1990) Induction of the *c-fos* proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Res.* 525, 256-266.
- Herdegen, T., Sandkuhler, J., Gass, P., Kiessling, M., Bravo, R. and Zimmermann, M. (1993) Jun, Fos, Krox, and CREB transcription factor proteins in the rat cortex: basal expression and induction by spreading depression and epileptic seizures. *J. Comp. Neurol.* 333, 271-288.
- Hernandez-Caceres, J., Macias-Gonzalez, R., Brozek, G. and Bures, J. (1987) Systemic ketamine blocks cortical spreading depression but does not delay the onset of terminal anoxic depolarization in rats. *Brain Res.* 437, 360-364.
- Herrera, D. G., Peterson, M. R. and Robertson, H. A. (1988) A calmodulin antagonist retards the development of kindling and reduces the production of c-Fos protein following a kindling stimulus. *Soc. Neurosci. Abstr.* 14, 228.1.
- Herrera, D. G., Figueiredo, B. F. and Cuello, A. C. (1993a) Differential regulation of *c-fos* expression after cortical brain injury during development. *Brain Res. Dev. Brain Res.* 76, 79-85.
- Herrera, D. G., Maysinger, D. and Gojny, M. (1993b) Induction of *c-fos* immunoreactivity in the hippocampus following potassium stimulation. *Neuroscience* 52, 237-244.
- Herrera, D. G., Maysinger, D., Gadiant, R., Boeckh, C., Otten, U. and Cuello, A. C. (1993c) Spreading depression induces *c-fos*-like immunoreactivity and NGF mRNA in the rat cerebral cortex. *Brain Res.* 602, 99-103.
- Herrera, D. G. and Robertson, H. A. (1989) Unilateral induction of c-Fos protein in cortex following cortical devascularization. *Brain Res.* 503, 205-210.
- Herrera, D. G. and Robertson, H. A. (1990a) Application of potassium chloride to the brain surface induces the *c-fos* proto-oncogene: reversal by MK-801. *Brain Res.* 510, 166-170.
- Herrera, D. G. and Robertson, H. A. (1990b) N-methyl-D-aspartate receptors mediate activation of the *c-fos* proto-oncogene in a model of brain injury. *Neuroscience* 35, 273-281.
- Hoffman, G. E., McDonald, T., Shedwick, R. and Nathanielsz, P. W. (1991) Activation of c-Fos in ovine fetal corticotropin-releasing hormone neurons at the time of parturition. *Endocrinology* 129, 3227-3233.
- Hoffman, G. E., Lee, W. S., Smith, M. S., Abbud, R., Roberts, M. M., Robinson, A. G. and Verbalis, J. G. (1993) c-Fos and Fos-related antigens as markers for neuronal activity: perspectives from neuroendocrine systems. *NIDA Res. Monogr.* 125, 117-133.
- Hooper, M. L., Chasson, B. J. and Robertson, H. A. (1994) Infusion of an antisense oligonucleotide to the immediate-early gene *c-fos* suppresses production of fos and produces a behavioural effect. *Neuroscience* 63, 917-924.
- Hope, B. T., Kelz, M. B., Duman, R. S. and Nestler, E. J. (1994) Chronic electroconvulsive seizure (ECS) treatment results in expression of a long-lasting AP-1 complex in brain with altered composition and characteristics. *J. Neurosci.* 14, 4318-4328.
- Hughes, P., Lawlor, P. and Dragunow, M. (1992) Basal expression of Fos, Fos-related, Jun, and Krox 24 proteins in rat hippocampus. *Brain Res. Mol. Brain Res.* 13, 355-357.
- Hunt, S. P., Pini, A. and Evan, G. (1987) Induction of *c-fos*-like protein in spinal cord neurons following sensory stimulation. *Nature* 328, 632-634.
- Ikeda, J., Nakajima, T., Gerfen, C. and Nowak, T. S. (1990) *In situ* hybridization analysis of *c-fos* and prodynorphin mRNA levels and distribution in gerbil hippocampus after transient ischemia. *Stroke suppl.* 21, 1-16.
- Ikeda, J., Nakajima, T., Osborne, O. C., Mies, G. and Nowak, T. S. (1994) Coexpression of *c-fos* and HSP70 mRNAs in gerbil brain after ischemia: induction threshold, distribution and time course evaluated by *in situ* hybridization. *Brain Res. Mol. Brain Res.* 26, 249-258.
- Imaki, T., Shibasaki, T., Hotta, M. and Demura, H. (1992) Early induction of *c-fos* precedes increased expression of corticotropin-releasing factor messenger ribonucleic acid in the paraventricular nucleus after immobilization stress. *Endocrinology* 131, 240-246.
- Imaki, T., Shibasaki, T., Hotta, M. and Demura, H. (1993) Intracerebroventricular administration of corticotropin-releasing factor induces *c-fos* mRNA expression in brain regions related to stress responses: comparison with pattern of *c-fos* mRNA induction after stress. *Brain Res.* 616, 114-125.
- Jacobson, L., Sharp, F. R. and Dallman, M. F. (1990) Induction of *c-fos*-like immunoreactivity in hypothalamic corticotropin-releasing factor neurons after adrenalectomy in the rat. *Endocrinology* 126, 1709-1719.
- Janik, D. and Mrosovsky, N. (1992) Gene expression in the geniculate induced by a nonphotic circadian phase shifting stimulus. *Neuroreport* 3, 575-578.
- Jensen, F. E., Firkusny, I. R. and Mower, G. D. (1993) Differences in *c-fos* immunoreactivity due to age and mode of seizure induction. *Mol. Brain Res.* 17, 185-193.
- Joh, T. H. and Weiser, M. (1993) Early and late molecular events occurring following neuronal degeneration in the dopamine system. *Adv. Neurol.* 60, 316-320.
- Jonec, V. and Wasterlain, C. G. (1979) Effect of inhibitors of protein synthesis on the development of kindled seizures in rats. *Exp. Neurol.* 66, 524-532.
- Jorgensen, M. B., Deckert, J., Wright, D. C. and Gehlert, D. R. (1989) Delayed *c-fos* proto-oncogene expression in the rat hippocampus induced by transient global cerebral ischemia: an *in situ* hybridization study. *Brain Res.* 484, 393-398.
- Jorgensen, M. B., Johansen, F. F. and Diemer, N. H. (1991) Post-ischemic and kainic acid-induced c-Fos protein expression in the rat hippocampus. *Acta Neurol. Scand.* 84, 352-356.
- Kamii, H., Kinouchi, H., Sharp, F. R., Epstein, C. J., Sagar, S. M. and Chan, P. H. (1994) Expression of *c-fos* mRNA after a mild focal cerebral ischemia in SOD-1 transgenic mice. *Brain Res.* 662, 240-244.
- Kilduff, T. S., Landel, H. B., Nagy, G. S., Sutin, E. L., Dement, W. C. and Heller, H. C. (1992) Melatonin influences Fos expression in the rat suprachiasmatic. *Brain Res. Mol. Brain Res.* 16, 47-56.
- Kjaer, A., Larsen, P. J., Knigge, U., Moller, M. and Warberg, J. (1994) Histamine stimulates *c-fos* expression in hypothalamic vasopressin-, oxytocin-, and corticotropin-releasing hormone-containing neurons. *Endocrinology* 134, 482-491.
- Koibuchi, N., Gibbs, R. B., Suzuki, M. and Pfaff, D. W. (1991) Thyroidectomy induces Fos-like immunoreactivity within thyrotropin-releasing hormone-expressing neurons located in the paraventricular nucleus of the adult rat hypothalamus. *Endocrinology* 129, 3208-3216.
- Kononen, J., Koistinaho, J. and Alho, H. (1990) Circadian rhythm in *c-fos*-like immunoreactivity in the rat brain. *Neurosci. Lett.* 120, 105-108.
- Kononen, J., Honkaniemi, J., Alho, H., Koistinaho, J., Iadarola, M. and Peltto-Huikko, M. (1992) Fos-like immunoreactivity in the rat hypothalamic-pituitary axis after immobilization stress. *Endocrinology* 130, 3041-3047.
- Kornhauser, J. M., Nelson, D. E., Mayo, K. E. and Takahashi, J. S. (1990) Photocircadian regulation of *c-fos* gene expression in the hamster suprachiasmatic nucleus. *Neuron* 5, 127-134.
- Kouzarides, T. and Ziff, E. (1988) The role of the leucine zipper in the Fos-Jun interaction. *Nature* 336, 646-651.
- Krukoff, T. L. (1993) Expression of *c-fos* in studies of central autonomic and sensory systems. *Mol. Neurobiol.* 7, 247-263.
- Kujubu, D. A., Lim, R. W., Varnum, B. and Herschman, H. R. (1987) Induction of transiently expressed genes in PC12 pheochromocytoma. *Oncogene* 1, 257-262.
- Leah, J. D., Sandkuhler, J., Herdegen, T., Murashov, A. and Zimmermann, M. (1992) Potentiated expression of fos protein in the rat spinal cord following bilateral noxious cutaneous stimulation. *Neuroscience* 48, 525-532.
- Leah, J. D., Herdegen, T., Murashov, A., Dragunow, M. and Bravo, R. (1993) Expression of immediate early gene proteins following axotomy and inhibition of axonal transport in the rat central nervous system. *Neuroscience* 57, 53-66.
- Leao, A. A. P. (1944) Spreading depression of activity in the cerebral cortex. *J. Neurophysiol.* 7, 359-390.
- Lee, J. H., Wilcox, G. L. and Beitz, A. J. (1992) Nitric oxide mediates Fos expression in the spinal cord induced by mechanical noxious stimulation. *Neuroreport* 3, 841-844.
- Lee, J. H., Price, R. H., Williams, F. G., Mayer, B. and Beitz, A. J. (1993) Nitric oxide synthase is found in some spinothalamic neurons and in neuronal processes that appose spinal neurons that express Fos induced by noxious stimulation. *Brain Res.* 608, 324-333.
- LeGalle LaSalle, G. (1988) Long lasting and sequential increases of c-Fos oncoprotein expression in kainic acid-induced status epilepticus. *Neurosci. Lett.* 88, 127-131.
- Lehmann, A., Isacson, H. and Hamberger, A. (1983) Effects of *in vivo* administration of kainic acid on the extracellular amino

- acid pool in the rabbit hippocampus. *J. Neurochem.* 40, 1314-1320.
- Lehmann, A., Hagberg, H., Jacobson, J. and Hamberger, A. (1985) Effects of state epilepticus on extracellular amino acids in the hippocampus. *Brain Res.* 359, 147-151.
- Lehmann, A., Sandberg, M. and Huxtable, R. J. (1986) *In vivo* release of neuroactive amines and aminoacids from the hippocampus of seizure resistant and seizure susceptible rats. *Neurochem. Intl* 8, 513-520.
- Lehmann, A. and Hamberger, A. (1983) Dihydro kainic acid affects extracellular taurine and phosphoethanolamine levels in the hippocampus. *Neurosci. Lett.* 38, 62-72.
- Leslie, R. A., Moorman, J. M. and Grahame-Smith, D. G. (1993a) Lithium enhances 5-HT_{2A} receptor-mediated *c-fos* expression in rat cerebral cortex. *Neuroreport* 5, 241-244.
- Leslie, R. A., Moorman, J. M., Coulson, A. and Grahame-Smith, D. G. (1993b) Serotonin₂/1C receptor activation causes a localized expression of the immediate-early gene *c-fos* in rat brain: evidence for involvement of dorsal raphe nucleus projection fibres. *Neuroscience* 53, 457-463.
- Lim, R. W., Varnum, B. C. and Herschman, H. R. (1987) Cloning of tetradecanoyl phorbol ester-induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. *Oncogene* 1, 263-270.
- Liu, P. K., Salminen, A., He, Y. Y., Jiang, M. H., Xue, J. J., Liu, J. S. and Hsu, C. Y. (1994) Suppression of ischemia-induced *fos* expression and AP-1 activity by an antisense oligodeoxynucleotide to *c-fos* mRNA. *Ann. Neurol.* 36, 566-576.
- Mack, K. J. and Mack, P. A. (1992) Induction of transcription factors in somatosensory cortex after tactile stimulation. *Mol. Brain Res.* 12, 141-147.
- Marranes, R., Willems, R., DePrins, E. and Wauquier, A. (1988) Evidence for a role of the *N*-methyl methyl-D-aspartate (NMDA) receptor in cortical spreading depression receptor in cortical spreading depression in the rat. *Brain Res.* 457, 226-240.
- McCormack, M. A., Rosen, K. M., Villa-Komaroff, L. and Mower, G. D. (1992) Changes in immediate early gene expression during postnatal development of cat cortex and cerebellum. *Brain Res. Mol. Brain Res.* 12, 215-223.
- McNaughton, L. A. and Hunt, S. P. (1992) Regulation of gene expression in astrocytes by excitatory amino acids. *Mol. Brain Res.* 16, 261-266.
- Mead, S., Ebling, F. J., Maywood, E. S., Humby, T., Herbert, J. and Hastings, M. H. (1992) A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of *c-fos* in the suprachiasmatic nuclei. *J. Neurosci.* 12, 2516-2522.
- Meister, B., Cortes, R., Villar, M. J., Schalling, M. and Hokfelt, T. (1990) Peptides and transmitter enzymes in hypothalamic magnocellular neurons after administration of hyperosmotic stimuli: comparison between messenger RNA and peptide/protein levels. *Cell Tissue Res.* 260, 279-297.
- Menetrey, D., Gannon, A., Levine, J. D. and Basbaum, A. I. (1989) Expression of *c-Fos* protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. *J. Comp. Neurol.* 285, 177-195.
- Milbrandt, J. (1986) Nerve growth factor rapidly induces *c-fos* mRNA in PC12 rat pheochromocytoma cells. *Proc. natn. Acad. Sci. U.S.A.* 83, 4789-4793.
- Milbrandt, J. (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* 238, 797-799.
- Miller, J. C. (1990) Induction of *c-fos* mRNA expression in rat striatum by neuroleptic drugs. *J. Neurochem.* 54, 1453-1455.
- Montarolo, P. G., Golet, P., Castelucci, V. F., Morgan, J., Kandel, E. R. and Schacher, S. (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* 234, 1249-1254.
- Morgan, J. I., Cohen, D. R., Hempstead, J. L. and Curran, T. (1987) Mapping patterns of *c-fos* expression in the central nervous system after seizure. *Science* 237, 192-197.
- Morgan, J. I. and Curran, T. (1986) Role of ion flux in the control of *c-fos* expression. *Nature* 322, 552-555.
- Morgan, J. I. and Curran, T. (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.* 14, 421-451.
- Morgan, P. F., Nadi, N. S., Karanian, J. and Linnoila, M. (1992) Mapping rat brain structures activated during ethanol withdrawal: role of glutamate and NMDA receptors. *Eur. J. Pharmacol.* 225, 217-223.
- Morris, S. W. and St. Claire, D. M. (1994) Eliminating *c-fos* as a candidate gene for early onset familial Alzheimer's disease. *Neurology* 44, 1762-1764.
- Moskowitz, M. A., Nozaki, K. and Kraig, R. P. (1993) Neocortical spreading depression provokes the expression of *c-Fos* protein-like immunoreactivity within trigeminal nucleus caudalis via trigemino-vascular mechanisms. *J. Neurosci.* 13, 1167-1177.
- Muller, R., Slamon, D. J., Tremblay, J. M., Cline, J. M. and Verma, I. M. (1982) Differential expression of cellular expression of cellular oncogenes during pre- and postnatal development of the mouse. *Nature* 299, 640-644.
- Muller, R., Verma, I. M. and Adamson, E. D. (1984) Expression of *c-onc* genes: *c-fos* transcripts accumulate to high levels during development of mouse placenta, yolk sac and amnion. *EMBO J.* 2, 679-687.
- Munell, F., Burke, R. E., Bandele, A. and Gubits, R. M. (1994) Localization of *c-fos*, *c-jun*, and HSP70 mRNA expression in brain after neonatal hypoxia-ischemia. *Brain Res. Dev. Brain Res.* 77, 111-121.
- Nakabeppu, Y., Ryder, K. and Nathans, D. (1988) DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* 55, 907-915.
- Nakabeppu, Y. and Nathans, D. (1991) A naturally occurring truncated form of Fos-B that inhibits Fos/Jun transcriptional activity. *Cell* 64, 751-759.
- Nakajima, T., Daval, J. L., Morgan, P. E. and Marangos, P. J. (1988) *C-fos* mRNA expression in brain induced by subconvulsive doses of caffeine. *Soc. Neurosci. Abstr.* 14, 463.1.
- Nakajima, T., Daval, J. L., Morgan, P. F., Post, R. M. and Marangos, P. J. (1989) Adenosinergic modulation of caffeine-induced *c-fos* mRNA expression in mouse brain. *Brain Res.* 501, 307-314.
- Nakamura, T., Datta, R., Kharbanda, S. and Kufe, D. (1991) Regulation of *jun* and *fos* gene expression in human monocytes by the macrophage colony-stimulating factor. *Cell Growth Diff.* 2, 267-272.
- Neumann-Haefelin, T., Wiessner, C., Vogel, P., Back, T. and Hossmann, K. A. (1994) Differential expression of the immediate early genes *c-fos*, *c-jun*, *jun-B*, and *NGFI-B* in the rat brain following transient forebrain ischemia. *J. Cereb. Blood Flow Metab.* 14, 206-216.
- Nikolaev, E., Werka, T. and Kaczmarek, L. (1992) *C-fos* proto-oncogene expression in rat brain after long-term training of two-way active avoidance reaction. *Behav. Brain Res.* 48, 91-94.
- Noguchi, K., Dubner, R. and Ruda, M. A. (1992) Preproenkephalin mRNA in spinal dorsal horn neurons is induced by peripheral inflammation and is co-localized with Fos and Fos-related proteins. *Neuroscience* 46, 561-570.
- Olenik, C., Lais, A. and Meyer, D. K. (1991) Effects of unilateral cortex lesions on gene expression of rat cortical cholecystokinin neurons. *Brain Res. Mol. Brain Res.* 10, 259-265.
- Onodera, H., Kogure, K., Ono, Y., Igarishi, K., Kiyota, Y. and Nagaoka, A. (1989) Proto-oncogene *c-fos* is transiently induced in the rat cerebral cortex after forebrain ischemia. *Neurosci. Lett.* 98, 101-104.
- Pang, Y., Kiba, H. and Jayaraman, A. (1993) Acute nicotine injections induce *c-fos* mostly in non-dopaminergic neurons of the midbrain of the rat. *Brain Res. Mol. Brain Res.* 20, 162-170.
- Park, H. T., Baek, S. Y., Kim, B. S., Kim, J. B. and Kim, J. J. (1993) Profile of Fos-like immunoreactivity induction by light stimuli in the intergeniculate leaflet is different from that of the suprachiasmatic nucleus. *Brain Res.* 610, 334-339.
- Paul, M. L., Graybiel, A. M., David, J. C. and Robertson, H. A. (1992) D1-like and D2-like dopamine receptors synergistically activate rotation and *c-fos* expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J. Neurosci.* 12, 3729-3742.
- Pezzone, M. A., Lee, W. S., Hoffman, G. E. and Rabin, B. S. (1992) Induction of *c-Fos* immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. *Brain Res.* 597, 41-50.
- Pezzone, M. A., Lee, W. S., Hoffman, G. E., Pezzone, K. M. and Rabin, B. S. (1993) Activation of brainstem catecholaminergic neurons by conditioned and unconditioned aversive stimuli as revealed by *c-fos* immunoreactivity. *Brain Res.* 608, 310-318.

- Popovici, T., Barbin, G. and Ben Ari, Y. (1988) Kainic acid-induced seizures increase c-Fos-like protein in the hippocampus. *Eur. J. Pharmacol.* **150**, 405-406.
- Pretel, S. and Piekut, D. T. (1991) Enkephalin, substance P, and serotonin axonal input to c-fos-like immunoreactive neurons of the rat spinal cord. *Peptides* **12**, 1243-1250.
- Racine, R. J. (1972) Modification of seizure activity by electrical stimulation: motor seizure. *Electroencephalogr. Clin. Neurophysiol.* **32**, 281-294.
- Raghupathi, R., Welsh, F. A., Lowenstein, D. H., Gennarelli, T. A. and McIntosh, T. K. (1995) Regional induction of c-fos and heat shock protein 72 mRNA following fluid-percussion brain injury in the rat. *J. Cereb. Blood Flow Metab.* **15**, 467-473.
- Rea, M. A. (1992) Different populations of cells in the suprachiasmatic nuclei express c-fos in association with light-induced phase delays and advances of the free-running activity rhythm in hamsters. *Brain Res.* **579**, 107-112.
- Rea, M. A., Buckley, B. and Lutton, L. M. (1993) Local administration of EAA antagonists blocks light-induced phase shifts and c-fos expression in hamster SCN. *Am. J. Physiol.* **265**, R1191-R1198.
- Richard, D., Rivest, S. and Rivier, C. (1992) The 5-hydroxytryptamine agonist fenfluramine increases Fos-like immunoreactivity in the brain. *Brain Res.* **594**, 131-137.
- Roberts, M. M., Robinson, A. G., Fitzsimmons, M. D., Grant, F., Lee, W. S. and Hoffman, G. E. (1993) C-fos expression in vasopressin and oxytocin neurons reveals functional heterogeneity within magnocellular neurons. *Neuroendocrinology* **57**, 388-400.
- Robertson, G. S. and Robertson, H. A. (1986) Synergistic effects of D1 and D2 dopamine agonists on turning behaviour in rats. *Brain Res.* **384**, 387-390.
- Robertson, G. S. and Robertson, H. A. (1989) Evidence that L-dopa-induced rotational behavior is dependent on both striatal and nigral mechanisms. *Journal of Neuroscience* **9**, 3326-3331.
- Robertson, G. S., Herrera, D. G., Dragunow, M. and Robertson, H. A. (1989a) L-Dopa activates c-fos in the striatum ipsilateral to a 6-hydroxydopamine lesion of the substantia nigra. *Eur. J. Pharmacol.* **159**, 99-100.
- Robertson, G. S., Vincent, S. R. and Fibiger, H. C. (1992) D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. *Neuroscience* **49**, 285-296.
- Robertson, H. A., Peterson, M. R., Murphy, K. and Robertson, G. S. (1989b) D1-dopamine receptor agonists selectively activate striatal c-fos independent of rotational behaviour. *Brain Res.* **503**, 346-349.
- Rogaev, E. I., Lukiw, W. J., Vaula, G., Haines, J. L., Rogaeva, E. A., Tsuda, T., Alexandrova, N., Liang, Y., Mortilla, M. and Amaducci, L. (1993) Analysis of the c-fos gene on chromosome 14 and the promoter of the amyloid precursor protein gene in familial Alzheimer's disease. *Neurology* **43**, 2275-2279.
- Rowe, D. W. and Erskine, M. S. (1993) C-fos proto-oncogene activity induced by mating in the preoptic area, hypothalamus and amygdala in the female rat: role of afferent input via the pelvic nerve. *Brain Res.* **621**, 25-34.
- Ruppert, C. and Wille, W. (1987) Proto-oncogene c-fos is highly inducible by disruption of neonatal but not mature brain tissue. *Mol. Brain Res.* **2**, 51-56.
- Rusak, B., McNaughton, L., Robertson, H. A. and Hunt, S. P. (1992) Circadian variation in photic regulation of immediate-early gene mRNAs in rat suprachiasmatic nucleus cells. *Brain Res. Mol. Brain Res.* **14**, 124-130.
- Ruzdijic, S., Pekovic, S., Kanazir, S., Ivkovic, S., Stojiljkovic, M. and Rakic, L. (1993) Temporal and spatial preferences of c-fos mRNA expression in the rat brain following cortical lesion. *Brain Res.* **601**, 230-240.
- Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder, K. and Baraban, J. M. (1988) Convulsant-induced increase in transcription factor messenger RNAs in rat brain. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7795-7799.
- Sagar, S. M., Sharp, F. R. and Curran, T. (1988) Expression of c-Fos protein in brain: metabolic mapping at the cellular level. *Science* **240**, 1328-1331.
- Sagar, S. M. and Sharp, F. R. (1990) Light induces a Fos-like nuclear antigen in retinal neurons. *Mol. Brain Res.* **7**, 17-21.
- Sambucetti, L. C. and Curran, T. (1986) The Fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. *Science* **234**, 1417-1419.
- Scammell, T. E., Price, K. J. and Sagar, S. M. (1993) Hyperthermia induces c-fos expression in the preoptic area. *Brain Res.* **618**, 303-307.
- Schreiber, S. S., Tocco, G., Najm, I., Finch, C. E., Johnson, S. A. and Baudry, M. (1992) Absence of c-fos induction in neonatal rat brain after seizures. *Neurosci. Lett.* **136**, 31-35.
- Schreiber, S. S., Najm, I., Tocco, G. and Baudry, M. (1993) Co-expression of HSP72 and c-fos in rat brain following kainic acid treatment. *Neuroreport* **5**, 269-272.
- Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M. and Aronin, N. (1994) Temporal regulation of light-induced Fos and Fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. *Neuroscience* **58**, 573-583.
- Sharp, F. R., Gonzalez, M. F., Hisanaga, K., Mobley, W. C. and Sagar, S. M. (1989) Induction of the c-fos gene product in rat forebrain following cortical lesions and NGF injections. *Neurosci. Lett.* **100**, 117-122.
- Sharp, F. R., Sagar, S. M., Hicks, K., Lowenstein, D. and Hisanaga, K. (1991) C-fos mRNA, Fos- and Fos-related antigen induction by hypertonic saline and stress. *J. Neurosci.* **11**, 2321-2331.
- Sharp, J. W., Sagar, S. M., Hisanaga, K., Jasper, P. and Sharp, F. R. (1990) The NMDA receptor mediates cortical induction of fos and fos-related antigens following cortical injury. *Expl Neurol.* **109**, 323-332.
- Shebab, S., Coffey, P., Dean, P. and Redgrave, P. (1992) Regional expression of fos-like immunoreactivity following seizures induced by pentylenetetrazol and maximal electroshock. *Expl Neurol.* **118**, 261-274.
- Shen, E., Dun, S. L., Ren, C., Bennett-Clarke, C. and Dun, N. J. (1992) Hypotension preferentially induces c-fos immunoreactivity in supraoptic vasopressin neurons [published erratum appears in *Brain Res.* (1992), 602(2), E1]. *Brain Res.* **593**, 136-139.
- Sheng, M. and Greenberg, M. E. (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477-485.
- Shin, C., McNamara, J. O., Morgan, J. I., Curran, T. and Cohen, D. R. (1990) Induction of c-fos mRNA expression by afterdischarge in the hippocampus of naive and kindled rats. *J. Neurochem.* **55**, 1050-1055.
- Simionato, M., Hosford, D. A., Labiner, D. M., Shin, C., Mansbach, H. H. and McNamara, J. O. (1991) Differential expression of immediate early genes in the hippocampus in the kindling model of epilepsy. *Brain Res. Mol. Brain Res.* **11**, 115-124.
- Smeyne, R. J., Curran, T. and Morgan, J. I. (1992a) Temporal and spatial expression of a fos-lacZ transgene in the developing nervous system. *Brain Res. Mol. Brain Res.* **16**, 158-162.
- Smeyne, R. J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T. and Morgan, J. I. (1992b) fos-lacZ transgenic mice: mapping sites of gene induction in the central nervous system. *Neuron* **8**, 13-23.
- Smeyne, R. J., Schilling, K., Oberdick, J., Robertson, L., Luk, D., Curran, T. and Morgan, J. I. (1993a) A fos-lacZ transgenic mouse that can be used for neuroanatomic mapping. *Adv. Neurol.* **59**, 285-291.
- Smeyne, R. J., Vendrell, M., Hayward, M., Baker, S. J., Miao, G. G., Schilling, K., Robertson, L. M., Curran, T. and Morgan, J. I. (1993b) Continuous c-fos expression precedes programmed cell death *in vivo* [published erratum appears in *Nature* (1993) 365(6443), 279]. *Nature* **363**, 166-169.
- Smith, M. A., Banerjee, S., Gold, P. W. and Glowa, J. (1992) Induction of c-fos mRNA in rat brain by conditioned and unconditioned stressors. *Brain Res.* **578**, 135-141.
- Sommer, W., Bjelke, B., Ganten, D. and Fuxe, K. (1993) Antisense oligonucleotide to c-fos induces ipsilateral rotational behaviour to D-amphetamine. *Neuroreport* **5**, 277-280.
- Sonnenberg, J. L., Mitchelmore, C., Macgregor-Leon, P. F., Hempstead, J., Morgan, J. I. and Curran, T. (1989) Glutamate receptor agonists increase the expression of Fos, FRA, and AP-1 DNA binding activity in the mammalian brain. *J. Neurosci. Res.* **24**, 72-80.
- Sprang, G. K. and Brown, I. R. (1987) Selective induction of a heat shock gene in fibre tracts and cerebellar neurons of the rabbit brain detected by *in situ* hybridization. *Mol. Brain Res.* **3**, 89-93.
- St-George-Hyslop, P., Haines, J., Rogaev, E., Mortilla, M., Vaula, G., Pericak-Vance, M., Foncin, J. F., Montesi, M., Bruni, A.,

- Sorbi, S., Rainero, I., Pinassi, L., Pollen, D., Polinsky, R., Nee, L., Kennedy, J., Macciardi, F., Rogaeva, E., Liang, Y., Alexandrova, N., Lukiw, W., Schlemper, K., Tanzi, R., Tsuda, T., Farren, L., Cantu, J.-M., Duara, R., Amaducci, L., Bergamini, L., Gusella, J., Roses, A. and Crapper McLachlan, D. (1992) Genetic evidence for a novel familial Alzheimer's disease locus on chromosome 14. *Nature Genetics* 2, 330-334.
- Stiles, C. D., Capone, G. T., Scher, C. D., Antoniadis, H. N., Van Wyke, J. J. and Pledger, W. J. (1979). *Proc. natn. Acad. Sci. U.S.A.* 76, 1279-1283.
- Stone, E. A., Zhang, Y., John, S. M. and Bing, G. (1991) *c-Fos* response to administration of catecholamines into brain by microdialysis. *Neurosci. Lett.* 133, 33-35.
- Stone, E. A., John, S. M., Bing, G. and Zhang, Y. (1992) Studies on the cellular localization of biochemical responses to catecholamines in the brain. *Brain Res. Bull.* 29, 285-288.
- Stone, E. A., Zhang, Y., John, S., Filer, D. and Bing, G. (1993) Effect of locus coeruleus lesion on *c-fos* expression in the cerebral cortex caused by yohimbine injection or stress. *Brain Res.* 603, 181-185.
- Sukhtame, V. P., Cao, X., Chang, L. C., Tsai-Morris, C., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., Le Beau, M. M. and Adamson, E. D. (1988) A zinc finger-encoding gene co-regulated with *c-fos* during growth and differentiation, and cellular depolarization. *Cell* 53, 37-43.
- Szekely, A. M., Barbaccia, M. L. and Costa, E. (1987) Activation of specific glutamate receptor subtypes increases *c-fos* proto-oncogene expression in primary cultures of cerebellar granule cells. *Neuropharmacology* 26, 1779-1782.
- Szekely, A. M., Barbaccia, M. L., Alho, H. and Costa, E. (1989) primary cultures of cerebellar granule cells the activation of *N*-methyl-D-aspartate-sensitive glutamate receptors induces *c-fos* mRNA expression. *Mol. Pharmacol.* 35, 401-408.
- Szekely, A. M., Costa, E. and Grayson, D. R. (1990) Transcriptional program coordination by *N*-methyl-D-aspartate-sensitive glutamate receptor stimulation in primary cultures of cerebellar neurons. *Mol. Pharmacol.* 38, 624-633.
- Szerb, J. C. (1991) Glutamate release and spreading depression in the fascia dentata in response to microdialysis with high K^+ : role of glia. *Brain Res.* 542, 259-265.
- Tischmeyer, W., Kaczmarek, L., Strauss, M., Jork, R. and Matthies, H. (1990) Accumulation of *c-fos* mRNA in rat hippocampus during acquisition of a brightness discrimination. *Behav. Neural Biol.* 54, 165-171.
- Tsujiro, T., Sano, H., Kubota, Y., Hsieh, S. T., Miyajima, T., Saito, K., Nakajima, M., Saito, N. and Yokoyama, M. (1992) Expression of Fos-like immunoreactivity by yohimbine and clonidine in the rat brain. *Eur. J. Pharmacol.* 226, 69-78.
- Uemura, Y., Kowall, N. W. and Beal, M. F. (1991) Global ischemia induce NMDA receptor-mediated *c-fos* expression in neurons resistant to injury in gerbil hippocampus. *Brain Res.* 542, 343-347.
- Vaccarino, F. M., Hayward, M. D., Nestler, E. J., Duman, R. S. and Tallman, J. F. (1992) Differential induction of immediate early genes by excitatory amino acid receptor types in primary cultures of cortical and striatal neurons. *Brain Res. Mol. Brain Res.* 12, 233-241.
- Van Beveren, C., van Straaten, F., Curran, T., Muller, R. and Verma, I. M. (1983) Analysis of FBJ-MuSV provirus and *c-fos* (mouse) gene reveals that viral and cellular fos gene products have different carboxy termini. *Cell* 32, 1241-1255.
- van Straaten, F., Muller, R., Curran, T., Van Beveren, C. and Verma, I. M. (1983) Complete nucleotide sequence of a human *c-onc* gene: deduced amino acid sequence of the human *c-Fos* protein. *Proc. natn. Acad. Sci. U.S.A.* 80, 3183-3187.
- Veening, J. G., van der Meer, M. J., Joosten, H., Hermus, A. R., Rijnkels, C. E., Geeraedts, L. M. and Sweep, C. G. (1993) Intravenous administration of interleukin-1 beta induces Fos-like immunoreactivity in corticotropin-releasing hormone neurons in the paraventricular hypothalamic nucleus of the rat. *J. Chem. Neuroanat.* 6, 391-397.
- Vendrell, M., Pujol, M. J., Tusell, J. M. and Serratos, J. (1992a) Effect of different convulsants on calmodulin levels and proto-oncogene *c-fos* expression in the central nervous system. *Brain Res. Mol. Brain Res.* 14, 285-292.
- Vendrell, M., Tusell, J. M. and Serratos, J. (1992b) *C-fos* expression as a model for studying the action of hexachlorocyclohexane isomers in the CNS. *J. Neurochem.* 58, 862-869.
- Vendrell, M., Tusell, J. M. and Serratos, J. (1992c) Effect of gamma-hexachlorocyclohexane and its isomers on proto-oncogene *c-fos* expression in brain. *Neurotoxicology* 13, 301-308.
- Verma, I. M., Curran, T., Muller, R., van Straaten, F., MacConell, W. F., Miller, A. D. and Van Beveren, C. (1984) The fos gene: organization and expression. In: *Cancer cells, 2. Oncogenes and Viral Genes*, p. 309. Eds W. G. F. Vande, A. J. Levine, W. C. Topp and J. D. Watson).
- Verma, I. M. (1987) Proto-oncogene *fos*: a multifaceted gene. In: *Oncogenes and Growth Factors*, p. 67. Eds R. A. Bradshaw and S. Prentis. Bks Demand: Ann Arbor, MI.
- Verrier, B., Muller, D., Bravo, R. and Muller, R. (1986) Wounding a fibroblast monolayer results in the rapid induction of the *c-fos* proto-oncogene. *EMBO J.* 5, 913-917.
- Wan, W., Janz, L., Vriend, C. Y., Sorensen, C. M., Greenberg, A. H. and Nance, D. M. (1993) Differential induction of *c-Fos* immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral administration of endotoxin. *Brain Res. Bull.* 32, 581-587.
- Wan, W., Wetmore, L., Sorensen, C. M., Greenberg, A. H. and Nance, D. M. (1994) Neural and biochemical mediators of endotoxin and stress-induced *c-fos* expression in the rat brain. *Brain Res. Bull.* 34, 7-14.
- Weaver, D. R., Rivkees, S. A. and Reppert, S. M. (1992) D1-dopamine receptors activate *c-fos* expression in the fetal suprachiasmatic nuclei. *Proc. natn. Acad. Sci. U.S.A.* 89, 9201-9204.
- Weiner, E. D., Kalasapudi, V. D., Papolos, D. F. and Lachman, H. M. (1991) Lithium augments pilocarpine-induced *fos* gene expression in rat brain. *Brain Res.* 553, 117-122.
- Weiser, M., Baker, H., Wessel, T. C. and Joh, T. H. (1993) Axotomy-induced differential gene induction in neurons of the locus ceruleus and substantia nigra. *Mol. Brain Res.* 17, 319-327.
- Welsh, F. A., Moyer, D. J. and Harris, V. A. (1992) Regional expression of heat shock protein-70 mRNA and *c-fos* mRNA following focal ischemia in rat brain. *J. Cereb. Blood Flow Metab.* 12, 204-212.
- Wessel, T. C., Joh, T. H. and Volpe, B. T. (1991) *In situ* hybridization analysis of *c-fos* and *c-jun* expression in the rat brain following transient forebrain ischemia. *Brain Res.* 567, 231-240.
- White, J. D. and Gall, C. M. (1987) Differential regulation of neuropeptide and proto-oncogene mRNA content in the hippocampus following recurrent seizures. *Mol. Brain Res.* 3, 21-29.
- Winston, S. M., Hayward, M. D., Nestler, E. J. and Duman, R. S. (1990) Chronic electroconvulsive seizures down-regulate expression of the immediate-early genes *c-fos* and *c-jun* in rat cerebral cortex. *J. Neurochem.* 54, 1920-1925.
- Wisden, W., Errington, M. L., Williams, S., Dunnet, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V. P. and Hunt, S. P. (1990) Differential expression of immediate early genes in the hippocampus and spinal cord. *Neuron* 4, 603-614.
- Young, S. T., Porrino, L. J. and Iadarola, M. J. (1991) Cocaine induces striatal *c-Fos*-immunoreactive proteins via dopaminergic D1 receptors. *Proc. natn. Acad. Sci. U.S.A.* 88, 1291-1295.
- Zhang, P., Hirsch, E. C., Damier, P., Duyckaerts, C. and Javoy-Agid, F. (1992a) *C-Fos* protein-like immunoreactivity: distribution in the human brain and over-expression in the hippocampus of patients with Alzheimer's disease. *Neuroscience* 46, 9-21.
- Zhang, W. Q., Pennypacker, K. R., Ye, H., Merchenthaler, I. J., Grimes, L., Iadarola, M. J. and Hong, J. S. (1992b) A 35 kDa Fos-related antigen is co-localized with substance P and dynorphin striatal neurons. *Brain Res.* 577, 312-317.
- Zhang, X., Ju, G. and Le Galle La Salle, G. (1991) Elevated expression of Jun and Fos-related proteins in transplanted striatal neurons. *Brain Res.* 558, 321-324.
- Zhang, Y., Van Reeth, O., Zee, P. C., Takahashi, J. S. and Turek, F. W. (1993) Fos protein expression in the circadian clock is not associated with phase shifts induced by a nonphotic stimulus, triazolam. *Neurosci. Lett.* 164, 203-208.
- Zinn, K., Keller, A., Whitmore, L. A. and Maniatis, T. (1988) 2-Aminopurine selectively inhibits the induction of b-interferon, *c-fos*, and *c-myc* gene expression. *Science* 240, 210-213.

IMENTARY

Signal integration at the *c-fos* promoter

Ralf Janknecht, Michael A. Cahill and Alfred Nordheim

Institute for Molecular Biology, Hannover Medical School,
D-30623 Hannover, Germany

Introduction

The *c-fos* gene encodes a basic region-leucine zipper transcription factor that requires heterodimerization with a member of the Jun family for stable DNA binding. Fos/Jun heterodimers are present in the AP-1 transcription factor complex and both c-Fos and c-Jun are capable of transforming cells (1). Continuous expression of *c-fos* can lead to aberrant differentiation (2) but may also sometimes block differentiation (3). Furthermore, antisense *c-fos* RNA or microinjected Fos-specific antibodies are able to inhibit cell growth (4-6). The study of the regulation of the *c-fos* gene may therefore provide important clues as to how cellular differentiation and proliferation are controlled.

The *c-fos* proto-oncogene is a member of the class of cellular immediate early genes which are rapidly and transiently induced upon stimulation of quiescent cells with growth factors or serum (7-9). In addition to these mitogens, many non-mitogenic signals such as UV-light (10) can also induce *c-fos* in a similar fashion. This transcriptional induction is independent of *de novo* synthesized proteins and inhibition of protein synthesis leads to an even stronger and prolonged induction of *c-fos* (11), indicating that components already exist for signal transduction cascades which target the *c-fos* promoter. Three proximal elements within the *c-fos* promoter have been identified as major targets for stimulating signals (Figure 1): the *sis* inducible element (SIE*) (12,13), the serum response element (SRE) (14,15) and the cAMP response element (CRE) (15). In the following, the pathways and mechanisms of signaling to these three promoter elements and their binding proteins will be discussed.

The *sis* inducible element (SIE)

The SIE has been shown to confer *c-fos* induction by conditioned medium from *v-sis* transformed cells. The platelet-derived growth factor (PDGF) B-chain gene is the cellular homolog of *v-sis*, and consistently the SIE is also responsive to PDGF (12,13). In addition, the SIE is involved in the stimulation of the *c-fos* gene by epidermal growth factor (EGF)

(16,17) as well as by interleukin-2 and -6 (18,19). However, PDGF, EGF and interleukin-2 also target the *c-fos* SRE (20-22), indicating that the SIE and the SRE can collaborate. It may even be that both the SIE and the SRE are required for *c-fos* upregulation in response to these agents as has been shown for interleukin-2 signaling via *p56^{lck}* (18), a protein tyrosine kinase of the Src family.

Inducible binding of the protein aggregate SIF (*sis* inducible factor) to the SIE has been observed *in vitro* and *in vivo* (12,23,24). Three different complexes of SIF can be resolved in gel retardation assays (16), of which the SIF-A and SIF-B complexes, but not the SIF-C complex, avidly interact with the native *c-fos* SIE (19). Subsequent analyses have indicated that two members of the Stat (signal transducer and activator of transcription) family form the SIF complexes: SIF-A is a homodimer of Stat3, SIF-C is a homodimer of Stat1α and SIF-B is a heterodimer of Stat1α and Stat3 (16,19,25).

Stat proteins were discovered as mediators of signaling induced by interferon-α (IFN-α) and interferon-γ (IFN-γ) (26). They are latent monomeric cytoplasmic transcription factors which dimerize and move into the cell nucleus upon tyrosine phosphorylation. The IFN-α response is mediated by ISGF3 (IFN-α-stimulated gene factor 3) which is composed of p48, of Stat2 (also called Stat113) and of either Stat1α (also called Stat91) or the C-terminal splice variant Stat1β (also called Stat84) (Figure 2A). The p48 protein mediates direct binding to an ISRE (interferon stimulation response element) and apparently 'piggy-backs' a heterodimer of Stat1 and Stat2. Since all known Stat proteins possess a Src homology region 2 (SH2) domain that can interact with phosphotyrosyl peptides, this heterodimerization may be mediated via reciprocal intermolecular SH2-phosphotyrosyl peptide interactions similar to the homodimerization of Stat1 (27). Furthermore, Stat1α and Stat1β homodimers can bind to an IFN-γ-activated site (GAS) and thereby mediate the IFN-γ response (Figure 2A). However, while the Stat1α dimer activates transcription, the Stat1β dimer apparently serves the function of a repressor. IFN-γ leads only to tyrosine phosphorylation of Stat1α and Stat1β. Thus, phosphorylated Stat2, which prevents homodimerization of

*Abbreviations: CaMK, Ca²⁺/calmodulin-dependent protein kinase; CBP, CREB binding protein; CRE, cAMP response element; CREB, CRE binding protein; EGF, epidermal growth factor; GAS, IFN-γ-activated site; IFN-α or -γ, interferon-α or -γ; ISGF3, IFN-α-stimulated gene factor 3; ISRE, interferon stimulation response element; Jak, Janus kinase; JNK, Jun kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEKK, MEK kinase; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; SAPK, stress-activated protein kinase; SH2 or SH3, Src homology region 2 or 3; SIE, *sis* inducible element; SIF, *sis* inducible factor; SRE, serum response element; SRF, serum response factor; Stat, signal transducer and activator of transcription; TCF, ternary complex factor; TPA, 12-O-tetradecanoylphorbol 13-acetate.

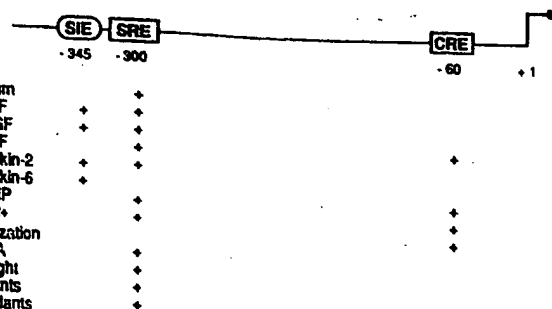


Fig. 1. Regulatory sequence elements within the human *c-fos* promoter. Examples of signals directed towards distinct sequence elements are given.

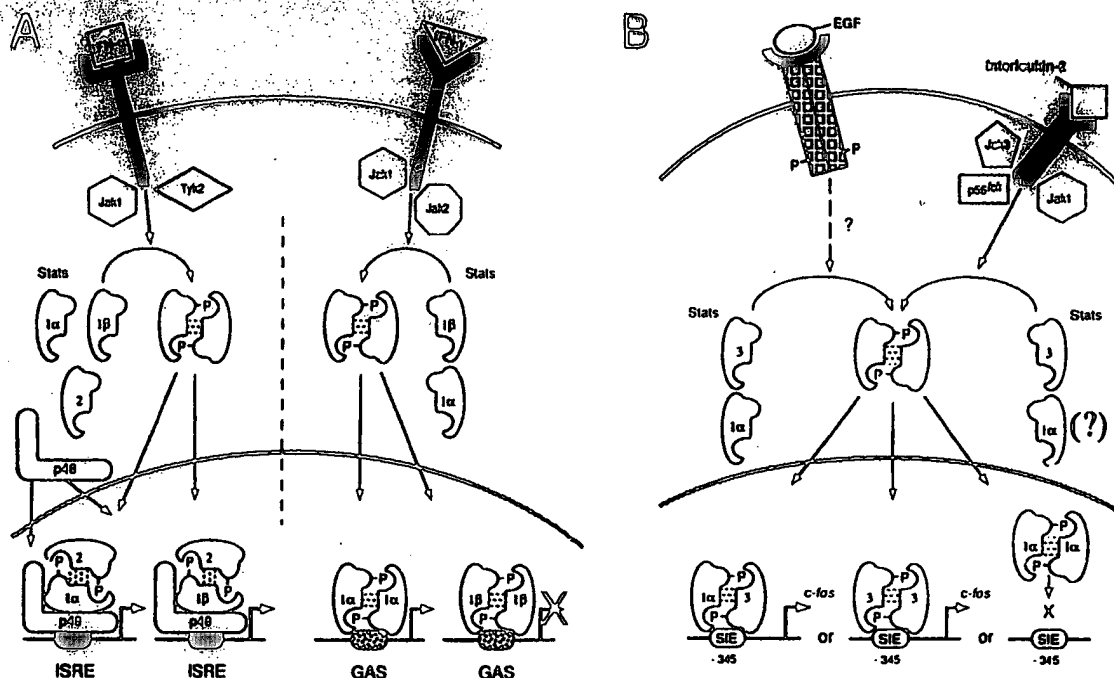


Fig. 2. Transcriptional regulation by Stats. (A) The classical interferon responses. (B) SIF-mediated induction of the *c-fos* SIE.

Stat1 by recruiting these factors into the ISGF3 complex, is only formed upon IFN- α , but not upon IFN- γ stimulation, thereby dictating the specificity of the interferon response (26). Tyrosine phosphorylation of Stats is proposed to be exerted by Jaks (Janus kinase or just 'another kinase'), which are characterized by the presence of two kinase-like domains, the more C-terminal of which possesses tyrosine kinase activity (28). While binding of IFN- α to its cognate receptor activates the tyrosine kinases Jak1 and Tyk2 but not Jak2, IFN- γ leads to the activation of Jak1 and Jak2 but not of Tyk2 (Figure 2A). However, it is presently unknown which of these kinases phosphorylates the different Stat proteins or whether other kinases are involved *in vivo*.

Figure 2B outlines how the Stat1 α and Stat3 proteins are activated and mediate SIE-dependent transcriptional upregulation. Binding of EGF to its cognate receptor leads to dimerization and tyrosine phosphorylation of the EGF receptor. One report suggests that Stat1 α interacts via its SH2 domain with the tyrosine-phosphorylated EGF receptor and thereby itself becomes phosphorylated by the kinase domain of the receptor (17). However, it is also possible that Stat1 α and Stat3 are phosphorylated by an unidentified tyrosine kinase which itself interacts with the activated EGF receptor. Activation of the SIE by interleukin-2 must involve such an intracellular tyrosine kinase because the interleukin-2 receptor lacks intrinsic kinase activity. One candidate is p56^{lck}, which has been shown to physically interact with a domain of the interleukin-2 receptor β chain (18,29). Another candidate is Jak1 (30) and a third is Jak3, which requires a different domain of the interleukin-2 receptor β chain in order to become activated (31). Finally, induction of the SIE does not always involve both the Stat3 homodimer and Stat1 α /Stat3 heterodimer as observed upon EGF stimulation. For instance, interleukin-6 stimulation only results in the formation of Stat3 homodimers, possibly because Stat1 α does not become phosphorylated (19).

The serum response element (SRE)

The SRE was originally demonstrated to mediate serum induction of the *c-fos* promoter (14). Serum can be regarded as a complex mixture of different growth factors and other mitogens, and individual growth factors such as PDGF (20) and EGF (21) can similarly induce *c-fos*. Cytokines like interleukin-2 (22) as well as the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) (10,20,21), UV-light (10), oxidants (32) and antioxidants (33) also activate the SRE.

The mechanism of growth factor-stimulated *c-fos* expression typically starts with the dimerization of growth factor receptor subunits upon ligand binding and their subsequent autophosphorylation on intracellular tyrosine residues (34). SH2 containing molecules can then interact with such phosphotyrosyl peptides and may themselves recruit other molecules to the activated growth factor receptor. The adapter molecule Grb2 not only contains an SH2 domain, but has in addition an SH3 (Src homology region 3) domain by which it interacts with a proline-rich domain of Sos (Figure 3). This protein is a guanine nucleotide exchange factor and catalyzes the transformation of the inactive GDP-Ras to the active GTP-Ras (35,36), which then recruits Raf-1 to the plasma membrane leading somehow to its activation (37,38). Apparently, Raf-1 can be activated in the absence of GTP-Ras provided that Raf-1 is localized to the plasma membrane (39,40), indicating that an unknown plasma membrane associated factor activates Raf-1. This factor may be the ζ isoform of protein kinase C (PKC) (41), which could cause activation of Raf-1 by direct phosphorylation. Furthermore, members of the 14-3-3 protein family may associate with Raf-1 and could thereby be involved in the activation of Raf-1 (42,43).

Phosphorylation of Raf-1 by PKC isoforms has been shown for PKC α , β and γ (44,45), all of which belong to the diacylglycerol-dependent PKC subclass in contrast to the ζ isoform (46,47). This explains the induction of *c-fos* by the

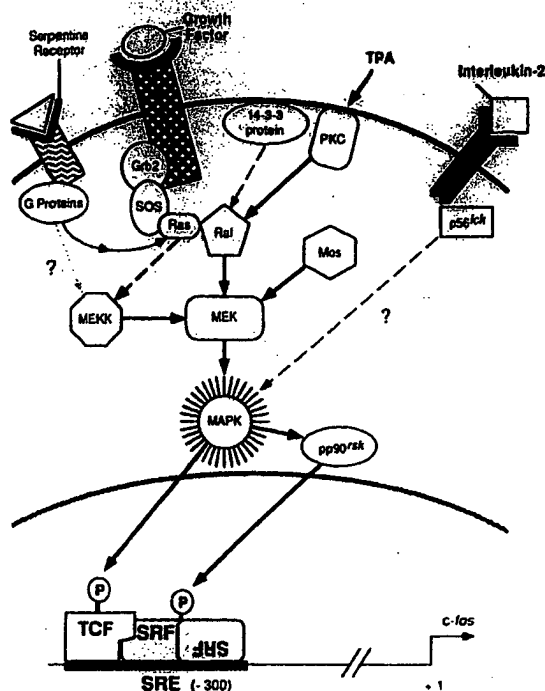


Fig. 3. Signal transduction via the *c-fos* SRE. While phosphorylation of TCFs results in enhanced *c-fos* transcription, phosphorylation of SRF has so far not been shown to be involved in *c-fos* regulation.

diacylglycerol analog TPA: after permeation of the plasma membrane, TPA interacts with and thereby activates diacylglycerol-dependent PKC isoforms, one or more of which may then initiate Raf-1-dependent signal transduction pathways (Figure 3).

Activated Raf-1 triggers a signal transduction cascade targeting the *c-fos* SRE (48,49) and is at the top of the mitogen-activated protein kinase (MAPK) pathway (Figure 3) (50–52). Raf-1 phosphorylates MEKs (MAPK kinases) which themselves are dual-specificity kinases phosphorylating both a threonine and a tyrosine in MAPKs (53). Several different MAPKs exist (54) and this complexity may even be enhanced by the family of the related Jun kinases (JNKs), also called stress-activated protein kinases (SAPKs) (55,56). Similarly, at least two functional MEKs have been identified in humans (57), and Raf-1 may be replaced by A-Raf or B-Raf (38,58).

In addition to Raf, MEK kinase (MEKK) and Mos can trigger the MAPK pathway (Figure 3) (59,60). While it is presently unclear how Mos itself is activated, MEKK is stimulated by growth factors in a Ras-dependent manner (61) and possibly also by G proteins (59), which are coupled to serpentine receptors (62). Additionally, some G proteins activate the MAPK pathway via Ras (63–65). Furthermore, MAPK has been shown to be phosphorylated and activated by p56^{lck} *in vitro* (66). Since p56^{lck} physically interacts with the interleukin-2 receptor β chain, it may be activated upon binding of interleukin-2 to its cognate receptor (18,29) and then directly stimulate MAPKs. Finally, oxidants, antioxidants or UV-light lead to the activation of MAPKs (67,68; J.Müller, M.A.C., P.Baeuerle and A.N., in preparation), although it is presently unknown how the MAPK pathway is triggered by these agents. However, Src, Ras and Raf-1 have been implicated in the activation of MAPK by UV-light.

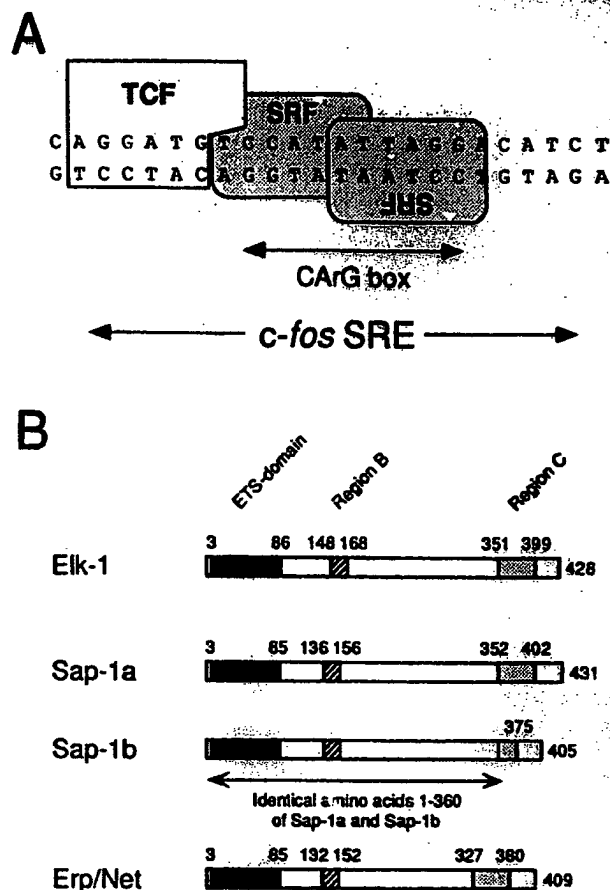


Fig. 4. The human *c-fos* SRE. (A) Interaction of one molecule TCF and a dimer of SRF with the SRE. The CCA³T₄GG 'CARG box' is the consensus binding site for SRF. (B) Structure of known TCFs. The ETS-domain mediates DNA binding, the homologous region B is required for interaction with SRF and the homologous region C contains critical MAPK phosphorylation sites. Coordinates refer to human Elk-1, human Sap-1a/Sap-1b and murine Erp/Net.

Once stimulated, MAPK enters the nucleus (69,70) and phosphorylates serine and threonine residues on various substrates including ternary complex factors (TCFs), thereby activating their potential to stimulate transcription (71–75). Only in conjunction with the serum response factor (SRF) do TCFs interact with the *c-fos* SRE (76) (Figure 4A). Since the *c-fos* SRE is occupied by TCF and SRF before, during and after induction (24,77), phosphorylation of TCFs may occur *in situ* at the *c-fos* promoter. However, it could also involve the rapid exchange of proteins bound to the promoter.

Several different TCFs have been identified (Figure 4B): Elk-1 (78), the two splice variants Sap-1a and Sap-1b (79) and Erp/Net (80,81). These proteins represent a subclass of the Ets family which is characterized by a DNA binding ETS-domain interacting with sites possessing a GGA³T₄ core sequence (82,83). TCFs are able to tightly bind certain Ets target sites (84,85) but not the *c-fos* SRE that contains an Ets binding site with a GGAT core sequence (Figure 4A). Only additional protein-protein contacts with SRE-bound SRF via the homologous region B allows stable binding of TCFs to the *c-fos* SRE (82,85–88).

TCFs feature a third homologous region at the C-terminus

(region C), which harbours several *in vivo* MAPK phosphorylation sites (89). Although there are more MAPK sites on both sides of region C, those within the region C most strongly affect transactivation (72–75). Since Sap-1b lacks the C-terminal half of region C, which appears to be indispensable for transactivation (87), it may represent a non-activatable TCF. Yet the functional diversity of TCFs is poorly understood. There is a certain tissue specific expression of TCFs, however in some organs all of them are expressed (81). It was suggested that distinct signal transduction pathways preferentially target different TCFs (90).

In contrast to TCFs, dimerized SRF can autonomously interact with the *c-fos* SRE (91). Several phosphorylation sites have been mapped within SRF (92–96), however only the one at Ser103 has been shown to be induced upon growth factor stimulation and could be phosphorylated by the kinase pp90^{rk} *in vitro* (97). Since pp90^{rk} is a target for MAPKs (98), future experiments should unravel whether inducible phosphorylation of SRF at Ser103 or other positions contributes to *c-fos* activation via the SRE.

Interestingly, TCF dephosphorylation coincides with the down-regulation of *c-fos* after its induction, and both TCF dephosphorylation and *c-fos* down-regulation are blocked in the presence of the protein phosphatase inhibitor okadaic acid (99). TCF dephosphorylation could be due to constitutive serine/threonine phosphatases such as protein phosphatase 2A (99) or potentially also to an inducible phosphatase. This would imply that a net phosphorylation of TCFs is only feasible when MAPKs are more active on TCFs than these phosphatases. MAPKs themselves are only transiently stimulated and their dephosphorylation can be caused by dual specificity (serine/threonine and tyrosine) phosphatases such as 3CH134/CL100 (100,101) or PAC1 (102). These protein phosphatases are inducible by stimuli known to also induce *c-fos* (100–102). Thus they could be responsible for the transient nature of MAPK activation, and consequently also for the transient phosphorylation of TCFs and the transient transcriptional induction of *c-fos*. Indeed, constitutively active PAC1 has been demonstrated to oppose the activating effect of MAPKs on the *c-fos* SRE (103).

Other mechanisms could also contribute to post-inductional repression of *c-fos*. One proposed mechanism is squelching via a low abundance coactivator necessary for induced transcription via the SRE, which could involve SRF or c-Fos itself (104). Both of these transcription factors are elevated in their concentration shortly after the transcriptional induction of *c-fos*, and both proteins have been shown to repress SRE-dependent transcription, c-Fos even independently of its DNA binding domain. Another proposed mechanism is that newly synthesized SRF may bind the SRE in a hypophosphorylated state, thereby contributing to *c-fos* down-regulation (105).

The cAMP response element (CRE)

The *c-fos* CRE represents a binding site for CREB (CRE binding protein) and related transcription factors. CREB contains a basic region-leucine zipper motif that is responsible for dimerization and DNA binding (106). Transactivation exerted by CREB requires phosphorylation on Ser133 (107) since only the phosphorylated form of CREB is capable of interaction with CBP (CREB binding protein), a coactivator thought to be required for the establishment of contacts with the transcriptional machinery (108–110). CREB is a well established target of protein kinase A (PKA) that itself is

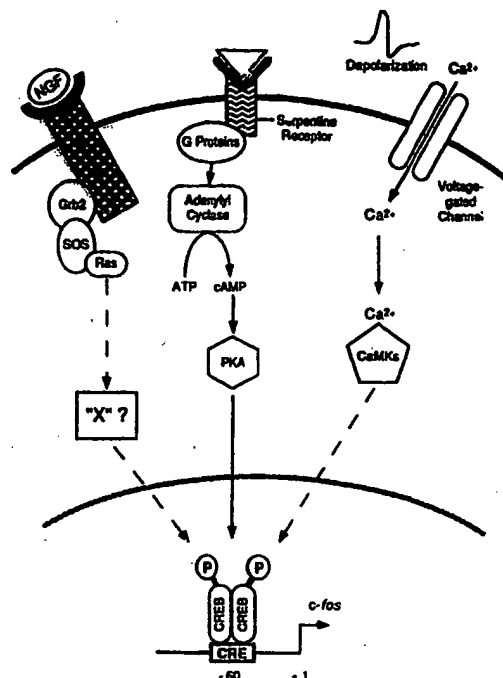


Fig. 5. Signal transduction pathways targeting the *c-fos* CRE. All signals have been demonstrated to induce phosphorylation of CREB on Ser133 leading to its activation. However note that hetero- or homodimers of other members of the CRE binding protein family might also activate *c-fos*.

activated by the second messenger cAMP (111). Accordingly, the *c-fos* CRE is involved in transcriptional upregulation in response to cAMP (112).

Signal transduction via PKA is often initiated at the plasma membrane upon interaction of a ligand (for instance the neurotransmitter nicotine) with a G protein-coupled serpentine receptor (Figure 5). This induces the dissociation of heterotrimeric G proteins into G α subunits and G β components leading to the activation of adenyl cyclase (62). Adenyl cyclase converts ATP into cAMP which interacts with the regulatory subunits of the inactive cytoplasmic form of PKA. cAMP binding results in the dissociation of the catalytic subunits of PKA, which can then translocate to the nucleus, phosphorylate CREB on Ser133 and thus activate CRE-dependent transcription (111). Similar to TCFs, the phosphorylation status of CREB determines the degree of transcriptional activity, and a constitutively active serine/threonine phosphatase (protein phosphatase 1) may dephosphorylate CREB *in vivo*, thereby attenuating CRE-driven transcription (113).

Membrane depolarization, which leads to an influx of Ca²⁺, results in the phosphorylation of CREB and the induction of *c-fos* via the CRE in neuronal PC12 cells. However, membrane depolarization does not significantly enhance intracellular cAMP levels, indicating that a signal transduction pathway independent of PKA is utilized (114). Several lines of evidence argue for the activation of Ca²⁺/calmodulin-dependent protein kinases (CaMKs) by Ca²⁺ influx and subsequent phosphorylation of CREB by CaMKs (Figure 5). (i) Depolarization of PC12 cells activates CaMKs (115). (ii) Both CaMK I and CaMK II are capable of phosphorylating CREB on Ser133 *in vitro* (116). (iii) *c-fos* induction by Ca²⁺ influx is abolished by calmodulin antagonists (117).

In addition, nerve growth factor (NGF) has been shown to

stimulate *c-fos* transcription in PC12 cells by phosphorylation of CREB on Ser133 which appears to be independent of PKA and CaMKs (Figure 5). Rather, an unknown Ras-dependent protein kinase seems to be involved (118). Since the NGF receptor is a classical growth factor receptor tyrosine kinase, it is likely that binding of its cognate ligand recruits Ras via Grb2 and Sos or other SH2-containing adaptor systems (36).

Redundant targeting of the *c-fos* promoter?

As described above, most signals targeting the *c-fos* SIE have been shown to also stimulate the SRE. Similarly, Ca^{2+} , cAMP and NGF not only activate the *c-fos* CRE but also the SRE (21,119,120). Why do signals often target more than one element in the *c-fos* promoter? One answer might be to ensure that even weak stimulants activate *c-fos*. Furthermore it appears that signaling to the *c-fos* promoter may nearly always involve the SRE, identifying this element as the pivotal regulatory sequence. However, in hippocampal neurons Ca^{2+} may activate two distinct signaling pathways depending on its mode of entry into neurons. Ca^{2+} entry via the NMDA receptor seems to activate preferentially the *c-fos* SRE and only very slightly the *c-fos* CRE, while this situation is reversed upon Ca^{2+} entry through a voltage-gated L-type Ca^{2+} channel (121). This example serves to illustrate that the other *c-fos* promoter elements may also function independently of a simultaneous activation of the SRE.

c-fos activation due to cAMP is apparently just as complex. The induction of PKA by cAMP, which leads to the activation of the *c-fos* CRE, has been shown to block the Ras-dependent activation of Raf-1 and thus the MAPK pathway in some cell lines, probably by inhibition of Raf-1 by PKA (122–124). This suggests that cAMP-mediated induction of *c-fos* could be mediated solely by the CRE, because any potential MAPK-mediated stimulation of the *c-fos* SRE would be attenuated. However, cAMP has been demonstrated to activate MAPK independently of Raf-1 in PC12 cells (125). This mechanism is thus apparently distinct from the Ras-dependent activation of MAPK induced by certain G proteins (63–65,126). It was speculated that this effect may be mediated by either action of PKA or by cAMP-gated ion channels. Therefore, the CRE and the SRE may also jointly up-regulate *c-fos* transcription in certain cell lines. Thus, signal transduction pathways targeting the *c-fos* promoter may be cooperative or antagonistic depending on the cell-type studied.

Biological function of the Fos protein

The Fos protein, as part of the AP-1 transcription factor complex, has been implicated in cell proliferation and transformation, particularly in the transition from the G_0 to G_1 phases of the cell-cycle (1,127). Yet homozygous *c-fos* knock-out mice, although displaying some defects in bone formation and the haematopoietic system, can mature to adulthood (128,129), indicating that c-Fos is not absolutely indispensable. Clearly, Fos overexpression in transgenic mice induces bone tumors (130), demonstrating the potential of *c-fos* to be oncogenic *in vivo*. However, Fos-induced transformation is not dependent on expression at particular stages of the cell cycle, rather it is related to the duration of Fos expression (131). These results suggest that the main function of Fos is not to regulate the expression of important determinants of the cell-cycle. Arguments for and against the involvement of c-Fos in cell-cycle control have been summarized by Hesketh (132).

Activation of signal transduction pathways variably changes patterns of gene expression and metabolism, often leading to greatly increased consumption of ATP. Mitochondrial ATP synthesis is accompanied by the generation of toxic reactive oxygen intermediates (133). Indeed, up to 2–5% of the oxygen reduced by mitochondria may be released to form H_2O_2 (134). Thus, increased metabolism following cell stimulation could induce redox stress. Interestingly, many of the genes induced by redox stress contain AP-1 binding sites which confer this responsiveness. This is the case from yeast to mammals (135–139), suggesting that one conserved and pivotal function of AP-1 transcription factors is the maintenance of intracellular redox homeostasis. This may explain why *c-fos* induction is coupled to almost any cellular stimulus: it may primarily serve to induce the provisional up-regulation of AP-1 stimulated redox stress enzymes, whose function is to detoxify the undesirable products of potential metabolic changes. How might this relate to Fos-mediated transformation? As well as inducing stress, reactive oxygen intermediates have been harnessed during evolution to activate signaling pathways as natural second messengers. This applies to both oxidative and reducing conditions (140). Some evidence exists to suggest that reactive oxygen intermediate levels can influence transformation. The activity of some enzymes of glutathione metabolism correlates well with a transforming phenotype (135,141). Furthermore redox stress can induce DNA-synthesis (32,142,143), and reactive oxygen levels are thought to play a role in neoplastic promotion (144). Thus, upsetting the natural regulation of reactive oxygen intermediate second messenger levels (potentially toward oxidative or reducing conditions), and the resulting perturbed expression of redox responsive genes, could facilitate the cell transformation induced by overexpressed Fos.

Acknowledgements

This work was supported by the Dr Mildred Scheel Stiftung, the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

- Curran,T. and Vogt,P.K. (1992) Dangerous liaisons: Fos and Jun, oncogenic transcription factors. In McKnight,S.L. and Yamamoto,K.R. (eds), *Transcriptional Regulation*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 797–831.
- Müller,R. and Wagner,E.F. (1984) Differentiation of F9 teratocarcinoma stem cells after transfer of *c-fos* proto-oncogenes. *Nature*, **311**, 438–442.
- Ito,E., Sonnenberg,J.L. and Narayanan,R. (1989) Nerve growth factor-induced differentiation in PC-12 cells is blocked by *fos* oncogene. *Oncogene*, **4**, 1193–1199.
- Holt,J.T., Gopal,T.V., Moulton,A.D. and Nienhuis,A.W. (1986) Inducible production of *c-fos* anti-sense RNA inhibits 3T3 cell proliferation. *Proc. Natl Acad. Sci. USA*, **83**, 4794–4798.
- Holt,J.T. (1993) Antisense rescue defines specialized and generalized functional domains for c-Fos protein. *Mol. Cell. Biol.*, **13**, 3821–3830.
- Riabowol,K.T., Bosatka,R.J., Ziff,E.B., Lamb,N.J. and Feramisco,J.R. (1988) Microinjection of Fos specific antibodies blocks DNA synthesis in fibroblast cells. *Mol. Cell. Biol.*, **8**, 1670–1677.
- Lau,L.F. and Nathans,D. (1985) Identification of a set of genes expressed during the G_0/G_1 transition of cultured mouse cells. *EMBO J.*, **4**, 3145–3151.
- Almendral,J.M., Sommer,D., MacDonald-Bravo,H., Burckhardt,J., Perera,J. and Bravo,R. (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell. Biol.*, **8**, 2140–2148.
- Bravo,R. (1990) Genes induced during the G_0/G_1 transition in mouse fibroblasts. *Cancer Biol.*, **1**, 37–46.
- Büscher,M., Rahmsdorf,H.J., Litfin,M., Karin,M. and Herrlich,P. (1988) Activation of the *c-fos* gene by UV and phorbol ester: Different signal transduction pathways converge to the same enhancer element. *Oncogene*, **3**, 301–311.

11. Greenberg, M.E., Hermanowski, A.L. and Ziff, E.B. (1986) Effect of protein synthesis inhibitors on growth factor activation of *c-fos*, *c-myc*, and actin gene transcription. *Mol. Cell. Biol.*, **6**, 1050-1057.
12. Hayes, T.E., Kitchen, A.M. and Cochran, B.H. (1987) Inducible binding of a factor to the *c-fos* regulatory region. *Proc. Natl. Acad. Sci. USA*, **84**, 1217-1225.
13. Wagner, B.J., Hayes, T.E., Hoban, C.J. and Cochran, B.H. (1990) The SIF binding element confers *sis*/PDGF inducibility onto the *c-fos* promoter. *EMBO J.*, **9**, 4477-4484.
14. Treisman, R. (1986) Identification of a protein-binding site that mediates transcriptional response of the *c-fos* gene to serum factors. *Cell*, **46**, 567-574.
15. Gilman, M.Z., Wilson, R.N. and Weinberg, R.A. (1986) Multiple protein-binding sites in the 5'-flanking region regulate *c-fos* expression. *Mol. Cell. Biol.*, **6**, 4305-4316.
16. Sadowski, H.B., Shuai, K., Darnell, J.E. and Gilman, M.Z. (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science*, **261**, 1739-1744.
17. Fu, X.-Y. and Zhang, J.-J. (1993) Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the *c-fos* gene promoter. *Cell*, **74**, 1135-1145.
18. Shibuya, H., Kohu, K., Yamada, K., Barsoumian, E.L., Perlmutter, R.M. and Taniguchi, T. (1994) Functional dissection of $p56^{lck}$, a protein tyrosine kinase which mediates interleukin-2-induced activation of the *c-fos* gene. *Mol. Cell. Biol.*, **14**, 5812-5819.
19. Zhong, Z., Wen, Z. and Darnell, J.E. (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, **264**, 95-98.
20. Siegfried, Z. and Ziff, E.B. (1989) Transcriptional activation by serum, PDGF, and TPA through the *c-fos* DSE: Cell type specific requirements for induction. *Oncogene*, **4**, 3-11.
21. Fisch, T.M., Prywes, R. and Roeder, R.G. (1987) *c-fos* sequences required for basal expression and induction by epidermal growth factor, 12-*O*-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. *Mol. Cell. Biol.*, **7**, 3490-3502.
22. Hatakeyama, M., Kawahara, A., Mori, H., Shibuya, H. and Taniguchi, T. (1992) *c-fos* gene induction by interleukin 2: Identification of the critical cytoplasmic regions within the interleukin 2 receptor β chain. *Proc. Natl. Acad. Sci. USA*, **89**, 2022-2026.
23. Sadowski, H.B. and Gilman, M.Z. (1993) Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature*, **362**, 79-83.
24. Herrera, R.E., Shaw, P.E. and Nordheim, A. (1989) Occupation of the *c-fos* serum response element *in vivo* by a multi-protein complex is unaltered by growth factor induction. *Nature*, **340**, 68-70.
25. Zhong, Z., Wen, Z. and Darnell, J.E. (1994) Stat3 and Stat 4: Members of the family of signal transducers and activators of transcription. *Proc. Natl. Acad. Sci. USA*, **91**, 4806-4810.
26. Darnell, J.E., Kerr, I.M. and Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415-1421.
27. Shuai, K., Horvath, C.M., Tsai, H., Huang, L.H., Qureshi, S.A., Cowburn, D. and Darnell, J.E. (1994) Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell*, **76**, 821-828.
28. Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B. and Silvennoinen, O. (1994) Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem. Sci.*, **19**, 222-227.
29. Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R.M. and Taniguchi, T. (1993) Association of $p56^{lck}$ with IL-2 receptor β chain is critical for the IL-2-induced activation of $p56^{lck}$. *EMBO J.*, **12**, 759-768.
30. Barber, D.L. and D'Andrea, A.D. (1994) Erythropoietin and interleukin-2 activate distinct JAK kinase family members. *Mol. Cell. Biol.*, **14**, 6505-6514.
31. Witthuhn, B.A., Silvennoinen, O., Miura, O., Lai, K.S., Cwik, C., Liu, E.T. and Ihle, J.N. (1994) Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. *Nature*, **370**, 153-157.
32. Nuse, K., Shibanuma, M., Kikuchi, K., Kageyama, H., Sakiyama, S. and Kuroki, T. (1991) Transcriptional activation of early-response genes by hydrogen peroxide in a mouse osteoblastic cell line. *Eur. J. Biochem.*, **201**, 99-106.
33. Meyer, M., Schreck, R. and Baeuerle, P.A. (1993) H_2O_2 and antioxidants have opposite effects on activation of NF- κ B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.*, **12**, 2005-2015.
34. Fantl, W.J., Johnson, D.E. and Williams, L.T. (1993) Signalling by receptor tyrosine kinases. *Annu. Rev. Biochem.*, **62**, 453-481.
35. Boguski, M.S. and McCormick, F. (1993) Proteins regulating Ras and its relatives. *Nature*, **366**, 643-654.
36. McCormick, F. (1993) How receptors turn Ras on. *Nature*, **363**, 15-16.
37. Moodie, S.A. and Wolfman, A. (1994) The 3Rs of life: Ras, Raf and growth regulation. *Trends Gen.*, **10**, 44-48.
38. Avruch, J., Zhang, X.-f. and Kyriakis, J.M. (1994) Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem. Sci.*, **19**, 279-283.
39. Leever, S.J., Paterson, H.F. and Marshall, C.J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature*, **369**, 411-414.
40. Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463-1467.
41. Berra, E., Diaz-Meco, M.T., Dominguez, I., Municio, M.M., Sanz, L., Lozano, J., Chapkin, R.S. and Moscat, J. (1993) Protein kinase C ζ isoform is critical for mitogenic transduction. *Cell*, **74**, 555-563.
42. Freed, E., Symons, M., Macdonald, S.G., McCormick, F. and Ruggieri, R. (1994) Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science*, **265**, 1713-1716.
43. Irie, K., Gotoh, Y., Yashar, B.M., Errede, B., Nishida, E. and Matsumoto, K. (1994) Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. *Science*, **265**, 1716-1719.
44. Süzer, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G.E. and Stabel, S. (1992) Activation of the c-Raf protein kinase by protein kinase C phosphorylation. *Oncogene*, **7**, 2259-2262.
45. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, R., Mischak, H., Finkenzeller, G., Marmé, D. and Rupp, U.R. (1993) Protein kinase C α activates Raf-1 by direct phosphorylation. *Nature*, **364**, 249-252.
46. Hug, H. and Sarre, T.F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.*, **291**, 329-343.
47. Dekker, L.V. and Parker, P.J. (1994) Protein kinase C—a question of specificity. *Trends Biochem. Sci.*, **19**, 73-77.
48. Kaibuchi, K., Fukumoto, Y., Oku, N., Hori, Y., Yamamoto, T., Toyoshima, K. and Takai, Y. (1989) Activation of the serum response element and 12-*O*-tetradecanoyl-13-acetate response element by the activated c-raf-1 protein in a manner independent of protein kinase C. *J. Biol. Chem.*, **264**, 20855-20858.
49. Jamal, S. and Ziff, E.B. (1990) Transactivation of *c-fos* and β -actin genes by raf as a step in early response to transmembrane signals. *Nature*, **340**, 463-466.
50. Dent, P., Haser, W., Haystead, T.A.J., Vincent, L.A., Roberts, T.M. and Sturgill, T.W. (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH3T3 cells and *in vitro*. *Science*, **257**, 1404-1407.
51. Howe, L.R., Leever, S.J., Gómez, N., Nakiely, S., Cohen, P. and Marshall, C.J. (1992) Activation of the MAP kinase pathway by the protein kinase raf. *Cell*, **71**, 335-342.
52. Kyriakis, J.M., App, H., Zhang, X.-f., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1992) Raf-1 activates MAP kinase-kinase. *Nature*, **358**, 417-421.
53. Marshall, C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82-89.
54. Pelech, S.L. and Sanghera, J.S. (1992) Mitogen-activated protein kinases: versatile transducers for cell signaling. *Trends Biochem. Sci.*, **17**, 233-238.
55. Dérjard, B., Hibi, M., Wu, I.-H., Barret, T., Su, B., Deng, T., Karin, M. and Davis, R.J. (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025-1037.
56. Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J. and Woodgett, J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156-160.
57. Zheng, C.-F. and Guan, K.-L. (1993) Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. *J. Biol. Chem.*, **268**, 11435-11439.
58. Jaiswal, R.K., Moodie, S.A., Wolfman, A. and Landreth, G.E. (1994) The mitogen-activated protein kinase cascade is activated by B-Raf in response to nerve growth factor through interaction with p21^{ras}. *Mol. Cell. Biol.*, **14**, 6944-6953.
59. Lange-Carter, C.A., Pleiman, C.M., Gardner, A.M., Blumer, K.J. and Johnson, G.L. (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science*, **260**, 315-319.
60. Nebreda, A.R., Hill, C., Gomez, N., Cohen, P. and Hunt, T. (1993) The protein kinase *mos* activates MAP kinase *in vitro* and stimulates the MAP kinase pathway in mammalian somatic cells *in vivo*. *FEBS Lett.*, **333**, 183-187.
61. Lange-Carter, C.A. and Johnson, G.L. (1994) Ras-dependent growth factor

- regulation of MEK kinase in PC12 cells. *Science*, **265**, 1458-1461.
62. Hepler, J.R. and Gillman, A.G. (1992) G-proteins. *Trends Biochem. Sci.*, **17**, 383-387.
 63. Gardner, A.M., Vaillancourt, R.R. and Johnson, G.L. (1993) Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase by G protein and tyrosine kinase oncoproteins. *J. Biol. Chem.*, **268**, 17896-17901.
 64. Howe, L.R. and Marshall, C.J. (1993) Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21^{ras} and p74^{raf-1}. *J. Biol. Chem.*, **268**, 20717-20720.
 65. Wintz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyer, L. and Johnson, G.L. (1993) Involvement of Ras and Raf in the G-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase and MAP kinase. *J. Biol. Chem.*, **268**, 19196-19199.
 66. Ettehadieh, E., Sanghera, J.S., Pelech, S.L., Hess-Bienz, D., Watts, J., Shastri, N. and Aebersold, R. (1992) Tyrosyl phosphorylation and activation of MAP kinases by p56^{lck}. *Science*, **255**, 853-855.
 67. Stevenson, M.A., Pollock, S.S., Coleman, C.N. and Calderwood, S.K. (1994) X-irradiation, phorbol esters, and H₂O₂ stimulate mitogen-activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates. *Cancer Res.*, **54**, 12-15.
 68. Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrlich, P. and Rahmsdorf, H.J. (1994) Involvement of growth factor receptors in the mammalian UVC response. *Cell*, **78**, 963-972.
 69. Chen, R.-H., Samecki, C. and Blenis, J. (1992) Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915-927.
 70. Lamy, F., Wilkin, F., Baptist, M., Posada, J., Roger, P.P. and Dumont, J.E. (1993) Phosphorylation of mitogen-activated protein kinases is involved in the epidermal growth factor and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway. *J. Biol. Chem.*, **268**, 8398-8401.
 71. Gille, H.G., Sharrocks, A.D. and Shaw, P.E. (1992) Phosphorylation of transcription factor p62^{TCF} by MAP kinase stimulates ternary complex formation at the *c-fos* promoter. *Nature*, **358**, 414-417.
 72. Hill, C.S., Marais, R., John, S., Wynne, J. and Treisman, R. (1993) Functional analysis of a growth factor-responsive transcription factor complex. *Cell*, **73**, 395-406.
 73. Janknecht, R., Ernst, W.H., Pingoud, V. and Nordheim, A. (1993) Activation of ternary complex factor Elk-1 by MAP kinases. *EMBO J.*, **12**, 5097-5104.
 74. Marais, R., Wynne, J. and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*, **73**, 381-393.
 75. Kortjenann, M., Thomae, O. and Shaw, P.E. (1994) Inhibition of *v-ras*-dependent *c-fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. *Mol. Cell. Biol.*, **14**, 4815-4824.
 76. Shaw, P.E., Schröter, H. and Nordheim, A. (1989) The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human *c-fos* promoter. *Cell*, **56**, 563-572.
 77. König, H. (1991) Cell-type specific multiprotein complex formation over the *c-fos* serum response element *in vivo*: ternary complex formation is not required for the induction of *c-fos*. *Nucleic Acids Res.*, **19**, 3607-3611.
 78. Hipskind, R.A., Rao, V.N., Mueller, C.G.F., Reddy, E.S.P. and Nordheim, A. (1991) Ets-related protein Elk-1 is homologous to the *c-fos* regulatory factor p62^{TCF}. *Nature*, **354**, 531-534.
 79. Dalton, S. and Treisman, R. (1992) Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell*, **68**, 597-612.
 80. Lopez, M., Oettgen, P., Akharali, Y., Dendorfer, U. and Libermann, T.A. (1994) ERP, a new member of the *ets* transcription factor/oncoprotein family: Cloning, characterization, and differential expression during B-lymphocyte development. *Mol. Cell. Biol.*, **14**, 3292-3309.
 81. Giovane, A., Pintzas, A., Maira, S.-M., Sobieszczuk, P. and Wasyluk, B. (1994) Net, a new *ets* transcription factor that is activated by Ras. *Genes Dev.*, **8**, 1502-1513.
 82. Treisman, R., Marais, R. and Wynne, J. (1992) Spatial flexibility in ternary complexes between SRF and its accessory proteins. *EMBO J.*, **11**, 4631-4640.
 83. Janknecht, R. and Nordheim, A. (1993) Gene regulation by Ets proteins. *Biochim. Biophys. Acta*, **1155**, 346-356.
 84. Rao, V.N. and Reddy, E.S.P. (1992) A divergent *ets*-related protein, Elk-1, recognizes similar *c-ets*-1 proto-oncogene target sequences and acts as a transcriptional activator. *Oncogene*, **7**, 65-70.
 85. Janknecht, R. and Nordheim, A. (1992) Elk-1 protein domains required for direct and SRF-assisted DNA-binding. *Nucleic Acids Res.*, **20**, 3317-3324.
 86. Rao, V.N. and Reddy, E.S.P. (1992) *elk-1* domains responsible for autonomous DNA binding, SRF-SRF interaction and negative regulation of DNA binding. *Oncogene*, **7**, 2335-2340.
 87. Janknecht, R., Zinck, R., Ernst, W.H. and Nordheim, A. (1994) Functional dissection of the transcription factor Elk-1. *Oncogene*, **9**, 1273-1278.
 88. Shore, P. and Sharrocks, A.D. (1994) The transcription factor Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. *Mol. Cell. Biol.*, **14**, 3283-3291.
 89. Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.*, **4**, 96-101.
 90. Hipskind, R.A., Büscher, D., Nordheim, A. and Baccarini, M. (1994) Ras/MAP kinase dependent and independent signaling pathways target distinct ternary complex factors. *Genes Dev.*, **8**, 1803-1816.
 91. Norman, C., Runswick, M., Pollock, R. and Treisman, R. (1988) Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the *c-fos* serum response element. *Cell*, **55**, 989-1003.
 92. Maniak, J.R. and Prywes, R. (1991) Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by casein kinase II. *Mol. Cell. Biol.*, **11**, 3652-3659.
 93. Janknecht, R., Hipskind, R.A., Houthaeve, T., Nordheim, A. and Stunnenberg, H.G. (1992) Identification of multiple SRF N-terminal phosphorylation sites affecting DNA binding properties. *EMBO J.*, **11**, 1045-1054.
 94. Marais, R.M., Hsuan, J.J., McGuigan, C., Wynne, J. and Treisman, R. (1992) Casein kinase II phosphorylation increases the rate of serum response factor-binding site exchange. *EMBO J.*, **11**, 97-105.
 95. Janknecht, R., Ernst, W.H., Houthaeve, T. and Nordheim, A. (1993) C-terminal phosphorylation of the serum-response factor. *Eur. J. Biochem.*, **216**, 469-475.
 96. Liu, S.-H., Ma, J.-T., Yueh, A.Y., Lees-Miller, S.P., Anderson, C.W. and Ng, S.-Y. (1993) The carboxyl-terminal transactivation domain of human serum response factor contains DNA-activated protein kinase phosphorylation sites. *J. Biol. Chem.*, **268**, 21147-21154.
 97. Rivera, V.M., Miranti, C.K., Misra, R.P., Ginty, D.D., Chen, R.-H., Blenis, J. and Greenberg, M.E. (1993) A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *Mol. Cell. Biol.*, **13**, 6260-6273.
 98. Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA*, **90**, 5889-5892.
 99. Zinck, R., Hipskind, R.A., Pingoud, V. and Nordheim, A. (1993) *c-fos* transcriptional activation and repression correlate temporally with the phosphorylation status of TCF. *EMBO J.*, **12**, 2377-2387.
 100. Charles, C.H., Abler, A.S. and Lau, L.F. (1992) cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. *Oncogene*, **7**, 187-190.
 101. Keyse, S.M. and Emslie, E.A. (1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature*, **359**, 644-647.
 102. Rohan, P.J., Davis, P., Moskaluk, C.A., Kearns, M., Krutzsch, H., Siebenlist, U. and Kelly, K. (1993) PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science*, **259**, 1763-1766.
 103. Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R.J. and Kelly, K. (1994) Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature*, **367**, 651-654.
 104. Cahill, M.A., Ernst, W.H., Janknecht, R. and Nordheim, A. (1994) Regulatory squelching. *FEBS Lett.*, **344**, 105-108.
 105. Misra, R.P., Rivera, V.M., Wang, J.M., Fan, P.D. and Greenberg, M.E. (1991) The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. *Mol. Cell. Biol.*, **11**, 4545-4554.
 106. Dwarki, V.J., Montminy, M. and Verma, I.M. (1990) Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. *EMBO J.*, **9**, 225-232.
 107. Gonzalez, G.A. and Montminy, M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*, **59**, 675-680.
 108. Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*, **365**, 855-859.
 109. Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J. and Montminy, M. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature*, **370**, 226-229.
 110. Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bächinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*, **370**, 223-229.

111. Lallé, E. and Sassone-Corsi, P. (1994) Signal transduction and gene regulation: the nuclear response to cAMP. *Biol. Chem.* **269**, 17359-17362.
112. Fisch, T.M., Thygesen, R., Simon, M.C. and Roper, R.G. (1989) Multiple sequence elements in the *c-fos* promoter mediate induction by cyclic AMP. *Genet. Dev.* **3**, 199-211.
113. Hagiwara, M., Alberts, A., Brindle, P., Meinhold, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S. and Montminy, M. (1992) Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell*, **70**, 105-113.
114. Sheng, M., McFadden, G. and Greenberg, M.E. (1990) Membrane depolarization and calcium induce *c-fos* transcription via phosphorylation of transcription factor CREB. *Neuron*, **4**, 571-582.
115. Griffith, L.C. and Shulman, H. (1988) The multi-functional calcium/calmodulin-dependent protein kinase mediates calcium-dependent phosphorylation of tyrosine hydroxylase. *J. Biol. Chem.* **263**, 9542-9549.
116. Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB: A Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*, **252**, 1427-1430.
117. Morgan, J.I. and Curran, T. (1986) Role of ion flux in the control of *c-fos* expression. *Nature*, **322**, 552-555.
118. Ginty, D.D., Bonni, A. and Greenberg, M.E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates *c-fos* transcription via phosphorylation of CREB. *Cell*, **77**, 713-725.
119. Bertowitz, L.A., Riabowol, K.T. and Gilman, M.Z. (1989) Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse *c-fos* promoter. *Mol. Cell. Biol.*, **9**, 4272-4281.
120. Visvader, J., Sassone-Corsi, P. and Verma, I.M. (1988) Two adjacent promoter elements mediate nerve growth factor activation of the *c-fos* gene and bind distinct nuclear complexes. *Proc. Natl. Acad. Sci. USA*, **85**, 9474-9478.
121. Bading, H., Ginty, D.D. and Greenberg, M.E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science*, **260**, 181-186.
122. Cook, S.J. and McCormick, F. (1993) Inhibition by cAMP of Ras-dependent activation of Raf. *Science*, **262**, 1069-1072.
123. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J. and Sturgill, T.W. (1993) Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science*, **262**, 1065-1069.
124. Häfner, S., Adler, H.S., Mischak, H., Jänosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M. and Kolch, W. (1994) Mechanism of inhibition of Raf-1 by protein kinase A. *Mol. Cell. Biol.*, **14**, 6696-6703.
125. Frödin, M., Peraldi, P. and Van Obberghen, E. (1994) Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. *J. Biol. Chem.*, **269**, 6207-6214.
126. Crespo, P., Xu, N., Simonds, W.F. and Gutkind, J.S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein β subunits. *Nature*, **369**, 418-420.
127. Angel, P. and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*, **1072**, 129-157.
128. Johnson, R.S., Spiegelman, B.M. and Papaioannou, V. (1992) Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene. *Cell*, **71**, 577-586.
129. Wang, Z.-Q., Oviatt, C., Grigoriadis, E., Möhle-Steinlein, U., Rüther, U. and Wagner, E.F. (1992) Bone and haematopoietic defects in mice lacking *c-fos*. *Nature*, **360**, 741-745.
130. Rüther, U., Komitowski, D., Schubert, F.R. and Wagner, E.F. (1989) *c-fos* expression induces bone tumors in transgenic mice. *Oncogene*, **4**, 861-865.
131. Miao, G.G. and Curran, T. (1994) Cell transformation by *c-fos* requires an extended period of expression and is independent of the cell cycle. *Mol. Cell. Biol.*, **14**, 4295-4310.
132. Hesketh, R. (1994) *The Oncogene Handbook*. Academic Press, London.
133. Halliwell, B. and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford.
134. Meister, A. (1991) Glutathione deficiency produced by inhibition of its synthesis, and its reversal: applications in research and therapy. *Pharmac. Ther.*, **51**, 155-194.
135. Daniel, V. (1993) Glutathione S-transferases: gene structure and regulation of expression. *Crit. Rev. Biochem. Molec. Biol.*, **28**, 173-207.
136. Sies, H. (1993) Strategies of antioxidant defense. *Eur. J. Biochem.*, **215**, 213-219.
137. Bergelson, S., Pinkus, R. and Daniel, V. (1994) Induction of AP-1 (Fos/Jun) by chemical agents mediates activation of glutathione S-transferase and quinone reductase gene expression. *Oncogene*, **9**, 565-571.
138. Kuge, S. and Jones, N. (1994) YAP1 dependent activation of *TRX2* is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.*, **13**, 655-664.
139. Wu, A.-L. and Moye-Rowley, W.S. (1994) *GSH1*, which encodes γ -glutamylcysteine synthetase, is a target gene for YAP-1 transcriptional regulation. *Mol. Cell. Biol.*, **14**, 5832-5839.
140. Pahl, H.L. and Bauerle, P.A. (1994) Oxygen and the control of gene expression. *BioEssays*, **16**, 497-502.
141. Sierra-Rivera, E., Meredith, M.J., Summar, M.L., Smith, M.D., Voorhees, G.J., Stoffel, C.M. and Freeman, M.L. (1994) Genes regulating glutathione concentrations in X-ray-transformed rat embryo fibroblasts: changes in γ -glutamylcysteine synthetase and γ -glutamyltranspeptidase expression. *Carcinogenesis*, **15**, 1301-1307.
142. Shibamura, M., Kuroki, T. and Nose, K. (1988) Induction of DNA replication and expression of proto-oncogene *c-myc* and *c-fos* in quiescent Balb/3T3 cells by xanthine/xanthine oxidase. *Oncogene*, **3**, 17-21.
143. Shibamura, M., Kuroki, T. and Nose, K. (1990) Stimulation by hydrogen peroxide of DNA synthesis, competence family gene expression and phosphorylation of a specific protein in quiescent Balb/3T3 cells. *Oncogene*, **5**, 1025-1032.
144. Cerutti, P.A. (1985) Prooxidant states and tumor promotion. *Science*, **227**, 375-381.

Received on October 21, 1994; revised on December 27, 1994; accepted on January 4, 1994

c-fos Is Required for Malignant Progression of Skin Tumors

Enrique Saez,* Susan E. Rutberg,†
Elisabetta Mueller,* Heather Oppenheim,*
Jennifer Smoluk,* Stuart H. Yuspa,†
and Bruce M. Spiegelman*

*Dana-Farber Cancer Institute
and Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

†Laboratory of Cellular Carcinogenesis
and Tumor Promotion
National Cancer Institute
Bethesda, Maryland 20892

Summary

The proto-oncogene *c-fos* is a major nuclear target for signal transduction pathways involved in the regulation of cell growth, differentiation, and transformation. Using the multistep skin carcinogenesis model, we have directly tested the ability of *c-fos*-deficient mice to develop cancer. Upon treatment with a tumor promoter, *c-fos* knockout mice carrying a *v-H-ras* transgene were able to develop benign tumors with similar kinetics and relative incidence as wild-type animals. However, *c-fos*-deficient papillomas quickly became very dry and hyperkeratinized, taking on an elongated, horny appearance. While wild-type papillomas eventually progressed into malignant tumors, *c-fos*-deficient tumors failed to undergo malignant conversion. Experiments in which *v-H-ras*-expressing keratinocytes were grafted onto nude mice suggest that *c-fos*-deficient cells have an intrinsic defect that hinders tumorigenesis. These results demonstrate that a member of the AP-1 family of transcription factors is required for the development of a malignant tumor.

Introduction

Advances in molecular biology have served to outline the multiple genetic components that are involved in the development of cancer. It is now well established that for a cell to grow aggressively within the context of the organism, proto-oncogenes must be mutated to become active growth inducers, tumor suppressor genes must be functionally lost, and the cell must be released from its own self-destruction program. Many of the important molecules in cancer development are proteins that function outside the nucleus of the cell, either assessing changes in the extracellular environment or relaying messages from sensor proteins to the control center of the cell. Because mitogens, growth factors, and tumor promoters regularly use these signal transduction pathways, mutations in genes implicated in either signal recognition or propagation can induce the cell to behave inappropriately. Upon reaching the nucleus, signals from these proteins must

communicate with transcription factors to elicit the changes in gene expression that can allow the cell to react to its new environment.

One class of transcription factors that it is thought to serve as the nuclear target of many oncogenic signal transduction pathways is the activator protein 1 (AP-1) family (reviewed by Ransone and Verma, 1990; Angel and Karin, 1991). This multigene group includes members of the Fos (*c-fos*, *fosB*, *fra-1*, and *fra-2*) and Jun (*c-jun*, *junB*, and *junD*) families. Members of the Fos family form heterodimers with Jun proteins and regulate transcription by binding DNA at AP-1 sites. These *cis*-acting regulatory elements are found in a variety of genes involved in cell proliferation, differentiation, and tumorigenesis. Oncogenic activation of several signal transduction pathways can result in increased AP-1 activity. For example, tumorigenic mutations in the proto-oncogene *H-ras* activate a family of mitogen-activated protein (MAP) kinases (JNKs/SAPKs) that phosphorylate Jun to augment its transactivation potential (Pulverer et al., 1991; Binetruy et al., 1991). Recently, an analogous class of Ras-responsive intracellular kinases has been described that seems to regulate Fos activity (Deng and Karin, 1994). It is unclear from these *in vitro* studies, however, how elevated AP-1-dependent gene expression can contribute to cancer development *in vivo*.

A clue to the significance of AP-1 activity for cell transformation can be found in the history of *fos* and *jun*, as both genes were initially described as the transforming principles of oncogenic retroviruses (Curran et al., 1982; Maki et al., 1987). Later studies using transgenic mice showed that stable expression of *c-fos* led to a dysregulation of bone growth eventually resulting in osteosarcomas and chondrosarcomas (Ruther et al., 1987, 1989). Transgenic mice expressing an oncogenic form of *jun* developed fibrosarcomas at sites of wound healing (Schuh et al., 1990). These observations indicated that aberrant expression of AP-1 genes could affect mitogenic control and promote neoplastic transformation of specific tissues.

Through the use of embryonic stem cell gene targeting technology, null mouse mutations of *c-fos* and *c-jun* have recently been generated (Johnson et al., 1992, 1993; Wang et al., 1992; Hilberg et al., 1993). While the *c-jun* knockout mutation is embryonic lethal, the *c-fos* null mouse is viable, though it displays a variety of tissue-specific anomalies. These defects include a severe form of osteopetrosis, a mild lymphopenia, delayed gametogenesis, and some behavioral abnormalities. In spite of these deficiencies, the availability of these mutant mice has allowed us to use them as tools to study the role of *c-fos* in cancer development *in vivo*. Our intention was to examine the unfolding of the neoplastic process in the absence of a critical component of the AP-1 family.

To address the relevance of *c-fos* for cancer development, we have used the classical model of multistep mouse skin carcinogenesis (reviewed by DiGiovanni, 1992; Hennings et al., 1993; Yuspa, 1994). Tumor devel-

opment in mouse skin can be divided into three steps: initiation, promotion, and progression. Initiation is an irreversible step that experimentally can be brought about by the application of a single dose of a mutagen (e.g., 2,5-dimethoxybenzaldehyde [DMBA]). Initiated cells can lie dormant until they are induced to proliferate, either by repeated treatments with a tumor promoter (e.g., 12-O-tetradecanoylphorbol-13-acetate [TPA]) or by a natural promoting stimulus such as wounding. During promotion, initiated cells are thought to have a growth advantage, and the tissue hyperplasia eventually results in a visible benign clonal outgrowth, a papilloma. Over time, papillomas can accumulate additional genetic mutations and progress into a malignant tumor, a squamous cell carcinoma. Carcinomas can undergo a further epithelial-mesenchymal transition to give rise to highly invasive spindle cell tumors (Klein-Szanto et al., 1989; Buchmann et al., 1991).

In this study, we have found that while *c-fos* does not seem to be necessary for normal epidermal differentiation or for the early proliferative steps of skin tumor formation, it is required for malignant tumor conversion. These results offer clear genetic evidence that an AP-1 factor is indispensable for the development of certain cancers.

Results

c-fos-Deficient Keratinocytes Form a Normal Epidermis

The skin is one of the few adult tissues in which *c-fos* expression has been reported to be constitutive (Fisher et al., 1991; Smeyne et al., 1992). Coupled with the observation that functional AP-1 sites can be found in a variety of genes associated with epidermal development, this finding raised the possibility that the skin of *c-fos* null mice could display an aberrant pattern of differentiation. To uncover any intrinsic abnormalities in unperturbed *c-fos*-deficient skin, we stained newborn epidermis with markers for specific stages of keratinocyte differentiation. Figure 1A shows double-labeled immunofluorescence staining for three markers: keratin 14 (K14), expressed in proliferating basal keratinocytes; keratin 1 (K1), induced in the differentiating spinous cells; and loricrin, a component of the cornified envelope expressed in the terminally differentiated cells of the granular layer. Comparison of the staining patterns for these and other epidermal differentiation markers failed to reveal any differences among mice of varying *c-fos* genotype. Histological examination of newborn *c-fos*-deficient skin showed that no obvious differ-

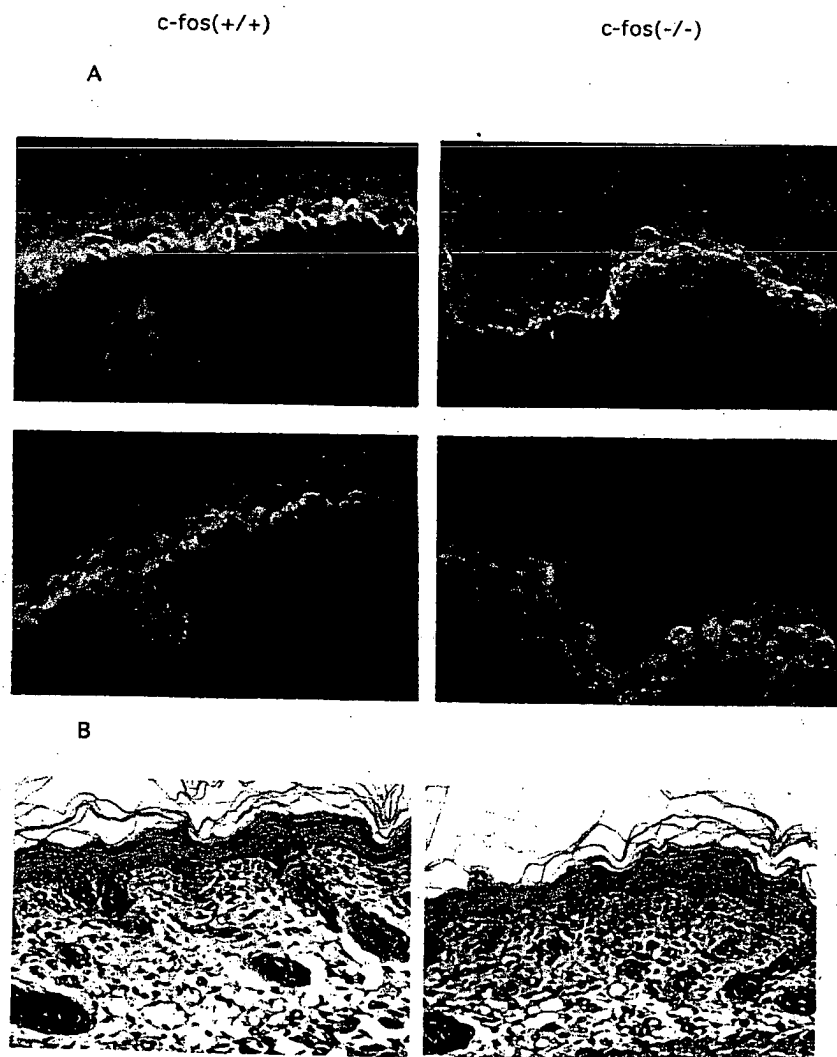


Figure 1. Analysis of Wild-Type and *c-fos*-Deficient Epidermis

(A) Indirect immunofluorescence staining of epidermal differentiation markers in newborn epidermis. In the top panels, K14 staining is shown in green and K1 in red. Areas of overlap appear yellow. The bottom panels show K14 in green and loricrin in red. Again, areas of common staining are yellow. Magnification, 204 \times .

(B) Hematoxylin and eosin staining of wild-type and *c-fos*-deficient newborn skin. Magnification, 163 \times .

ences exist in epidermal organization or cellularity (Figure 1B). Analogous results were obtained with thinner adult skin. Furthermore, when newborn mice were injected with bromodeoxyuridine to identify epidermal cells undergoing DNA replication, the number of proliferating cells was found to be similar in both genotypes (data not shown). These observations allowed us to conclude that, on the basis of keratinocyte growth rate or pattern of differentiation-dependent gene expression, the skin of *c-fos*-deficient mice is indistinguishable from that of wild-type mice.

c-fos Is Not Required for the Early Stages of Skin Carcinogenesis

To induce tumor formation in the skin of mice, we used a modified version of a traditional DMBA/TPA tumor induction protocol. Instead of using a chemical initiator, *c-fos* mutant mice were bred with a transgenic strain (TG.AC) carrying a v-H-ras transgene that can act as a classical initiation event (Leder et al., 1990). The existence of this transgenic strain enabled us to avoid the use of initiators that would have introduced undefined genetic mutations, potentially complicating the interpretation of the results. Even though the transgene is under the control of an embryonic globin promoter and thus is not normally active in the skin, expression of the transgene is induced in epidermal cells when they are prompted to proliferate either by application of tumor promoters or by a natural stimulus such as hair plucking (Hansen and Tennant, 1994a, 1994b). Mice carrying the TG.AC transgene are very sensitive to a variety of tumor promoters; they rapidly develop large numbers of papillomas (Spalding et al., 1993).

Mice homozygous for the TG.AC transgene were mated with mice heterozygous for the *c-fos* null mutation. Offspring carrying both mutations were identified and interbred to generate mice of all possible genotypes. The dorsal epidermis of these mice was then treated with the tumor promoter TPA twice a week for 5 weeks, according to standard promotion protocols for the TG.AC strain. Treated mice were monitored biweekly for the appearance of papillomas. As can be discerned from Figure 2, *c-fos*-deficient mice were able to develop papillomas, and they did so with equivalent kinetics to those of their wild-type and heterozygous littermates. Furthermore, the time to appearance of the first tumor and the number of papillomas per mouse were indistinguishable among genotypes. Because papillomas are very heterogenous and often tend to coalesce with other papillomas, size comparisons are difficult. Nevertheless, no obvious differences in tumor size could be observed among mice of differing genotypes.

Table 1 summarizes benign tumor incidence rates. A small minority of v-H-ras transgenic mice in each group proved to be resistant to TPA treatment. This resistance did not correlate with *c-fos* genotype and has previously been described for inbred FvB/N TG.AC mice. Two conclusions can be drawn from the data in Table 1 regarding the role of *c-fos* in the early steps of mouse skin carcinogenesis. First, the absence of *c-fos* cannot act as an initial-

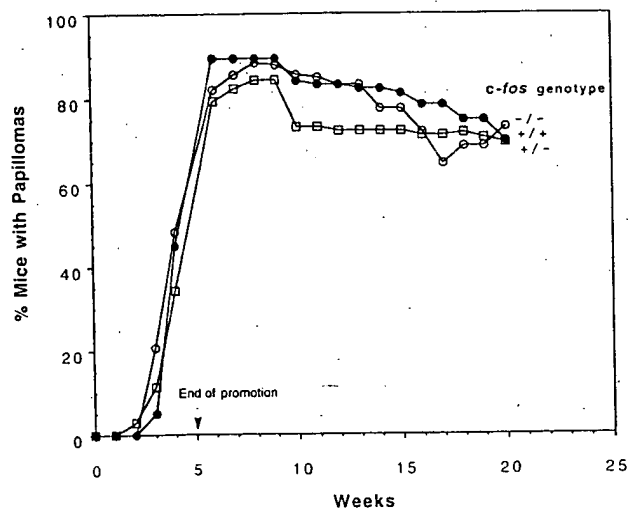


Figure 2. Initial Kinetics of Papilloma Appearance

Number of papilloma-bearing mice as a percentage of those treated with tumor promoter: wild-type mice (closed circles; $n = 19$), heterozygous mice (open squares; $n = 35$), and *c-fos* null mice (open circles; $n = 29$).

Second, since *c-fos*-deficient mice bearing the v-H-ras transgene developed papillomas in a normal manner, it is clear that in this model of carcinogenesis, this proto-oncogene is not necessary for promotion.

c-fos-Deficient Papillomas Evolve into Horny, Elongated Tumors Characterized by Massive Hyperkeratinization

Although *c-fos*-deficient papillomas initially displayed an identical external appearance to wild-type tumors, a striking change in morphology was noticed soon after the end of promotion. Within 4 weeks after TPA treatment was completed, the papillomas on all *c-fos* null mice started to become very dry, elongated, and hyperkeratinized. This change in morphology was very apparent as the study advanced. Though some papilloma regression was observed in mice of all genotypes, the papillomas that persisted on wild-type and heterozygous mice remained vigorous and well vascularized. In contrast, mutant papillomas became desiccated and extremely hyperkeratinized and showed little external vascularization. Figure 3A shows a comparison of wild-type and *c-fos* null papillomas soon after the end of promotion. While wild-type papillomas retained a similar morphology until the end of the study, *c-fos*-deficient tumors acquired a rather horny appearance: they evolved into severely keratinized, grotesque projections. Examples of these older *c-fos*-deficient tumors are shown in Figure 3C. Histopathology of wild-type and mutant papillomas revealed that *c-fos*-deficient tumors indeed display a very dramatic hyperkeratinization, showing a remarkable increase in the terminally differentiated stratum corneum (Figure 4). In addition, *c-fos*-deficient papillomas seemed to present an abrupt transition between the basal layer and the stratum corneum.

Table 1. Benign Tumor Incidence Rates

Genotype	Treatment	Papillomas	Incidence (%)	Papillomas per Mouse	Time to First Tumor
<i>c-fos</i> wild-type transgenic v-H-ras	TPA	17 of 19	90	26 ± 14.4	33.4 ± 7.2
	Acetone	0 of 4	0		
<i>c-fos</i> heterozygous transgenic v-H-ras	TPA	30 of 35	86	22 ± 15.7	34.8 ± 8.9
	Acetone	0 of 5	0		
<i>c-fos</i> -deficient transgenic v-H-ras	TPA	26 of 29	90	23 ± 14.3	32.0 ± 7.7
	Acetone	0 of 6	0		
<i>c-fos</i> wild type	TPA	0 of 9	0		
<i>c-fos</i> heterozygous	TPA	0 of 13	0		
<i>c-fos</i> deficient	TPA	0 of 6	0		

At 12 weeks of age, all mice were shaved and promotion treatment was started either with TPA or with solvent alone (acetone). Mice were monitored biweekly for the appearance of tumors. Papillomas per mouse and time to first tumor statistics are given as mean ± SD.

the *c-fos* null mutation appeared identical to wild-type papillomas: their morphology did not change, and they did not present an abnormal epidermal architecture.

To investigate whether these hyperkeratinized *c-fos*-deficient tumors showed an abnormal rate of regression, we compared the average number of papillomas per mouse at 9 weeks and at 23 weeks. Since TPA treatment was discontinued at the end of week 5, the majority of regressing papillomas should have disappeared by week 23. In wild-type mice, the mean number of papillomas per mouse decreased from 26 at week 9 to 10.5 at week 23 (a 60% reduction). Heterozygous mice showed a very similar decline, from 22.1 tumors per mouse at week 9 to 8.9 at week 23 (a 60% decrease). The mean number of papillomas per mouse in *c-fos*-deficient animals decreased from 23 at week 9 to 4.7 at week 23 (an 80% reduction). These data show that *c-fos* is not required for the persistence of papillomas. Whether papilloma regression rates are significantly affected by the *c-fos* null mutation is an issue that will require more study.

Papillomas Lacking *c-fos* Fail to Undergo Malignant Conversion

Wild-type mice began to develop malignant skin tumors around week 20, although most cases of malignant progression were noticed between weeks 25 and 30. Malignant lesions first appeared as ulcerated areas within a papilloma that grew rapidly. These malignant tumors were identified histologically as either squamous cell carcinomas or, more frequently, spindle cell tumors, in agreement with what has been described for the TG.AC strain (French et al., 1994). By the end of the study (week 45), malignant progression had been observed in 4 of 7 (57%) surviving wild-type mice and in 7 of 17 (41%) heterozygous mice (Table 2). In contrast, no malignant skin tumors had been detected in 13 remaining *c-fos* null mice. A detailed pathological examination of all remaining *c-fos*-deficient papillomas at the end of the study failed to find any evidence of microscopic malignant progression. The decrease in the number of mice that were available at the later timepoint to assess progression is a reflection of the fact that the v-H-ras TG.AC transgene induces a variety of other tumors

(e.g., hematopoietic malignancies) that necessitate the sacrifice of the affected animal. Nevertheless, the difference in progression rates of tumors with or without *c-fos* (57% and 41% versus 0%) is statistically significant ($p < 0.0001$) and allows us to conclude that *c-fos* is required for malignant progression of experimentally induced skin tumors. Wild-type and *c-fos*-deficient papillomas expressed similar levels of transgenic v-H-ras 1 month after the end of tumor promoter treatment (Figure 5B). Hence, the inability of *c-fos*-deficient papillomas to become malignant is not due to a reduced level of oncogenic H-ras expression. These final progression figures do not exclude the possibility of a heterozygous effect, although the finding that heterozygous papillomas behave like wild-type papillomas by all criteria examined argues against it.

Alterations in Gene Expression in *c-fos* Null Papillomas

To examine whether the lack of malignant progression of *c-fos*-deficient tumors correlated with specific changes in gene expression, we isolated RNA from pools of papillomas from wild-type and *c-fos*-deficient mice. Each pool consisted of five to six tumors taken 1 month after the end of TPA treatment. These RNAs were used to measure the degree of expression of certain AP-1-regulated genes in the papillomas. The genes encoding the tumor metalloproteases stromelysin and type I collagenase were of particular interest because studies using *c-fos*-deficient fibroblasts had shown that *c-fos* is required for the induction of these genes in response to mitogenic stimulation (Hu et al., 1994). In addition, enhanced expression of these tumor metalloproteases has been associated with the progression of benign, encapsulated papillomas to malignant, invasive tumors (Matrisian et al., 1986; Liotta and Stetler-Stevenson, 1990). While the stromelysin and type I collagenase mRNAs were detectable in the pools of wild-type tumors, they were virtually absent in RNA pools from *c-fos*-deficient papillomas (Figures 5A and 5B). The apparent lack of external vascularity of mutant tumors also prompted us to assess the level of expression of angiogenic factors in the papillomas. Of special interest was the pattern of expression of vascular endothelial growth

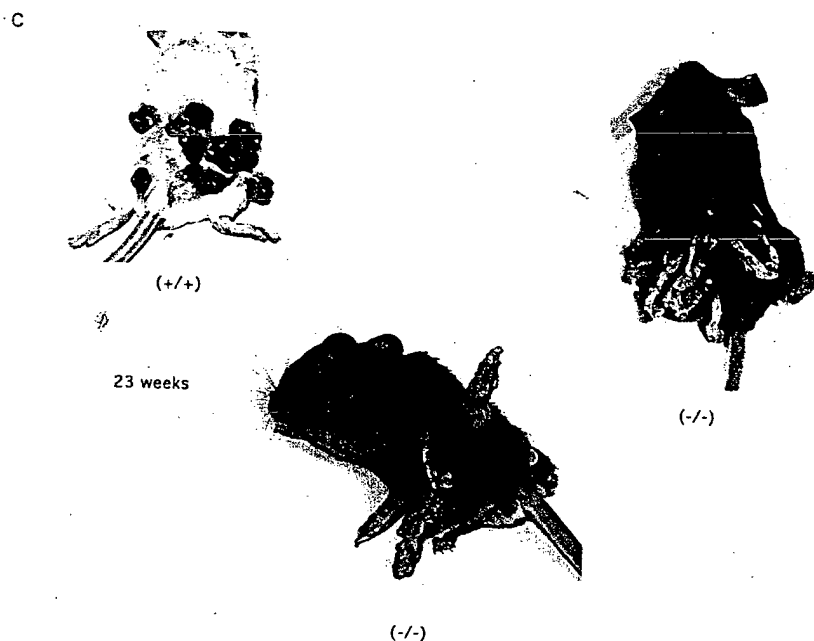
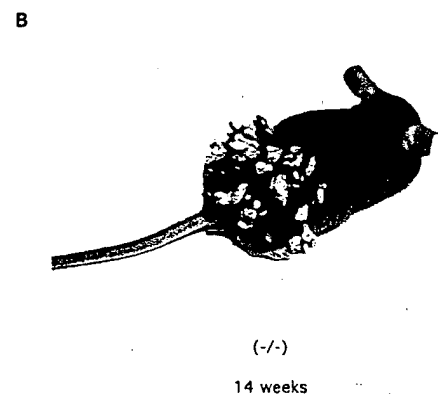
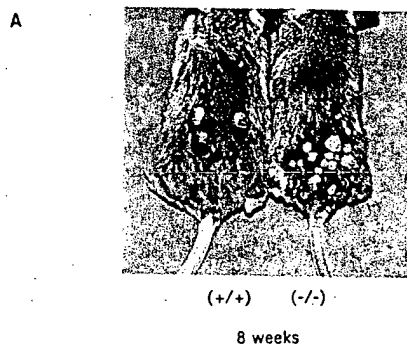


Figure 3. External Appearance of Papillomas

(A) A wild-type mouse (left) and a *c-fos*-deficient littermate (right) are shown 3 weeks after the end of promotion.

(B) *c-fos* null mouse 9 weeks after the end of promotion (14 weeks from the start of treatment); note the change in morphology that is now obvious in all papillomas.

(C) Wild-type and *c-fos*-deficient tumors 4 months after the conclusion of TPA treatment. The black mouse is the same mouse as that shown in (B). Notice the absence of external vascularity in the large tumors of the brown *c-fos*-deficient mouse. Color coat segregates independently of both the TG.AC transgene and the *c-fos* null mutation.

factor (VEGF), for this protein may be the prime regulator of normal and tumor angiogenesis (Klagsbrun and Soker, 1993). The VEGF gene was expressed in *c-fos*-deficient tumors, but the levels of VEGF mRNA measured in mutant papillomas were 5- to 10-fold lower than those found in RNA pools derived from wild-type papillomas (Figure 5C).

The changes in keratin gene expression that take place during a protocol of skin carcinogenesis are well established and can often serve as markers of the degree of tumor progression. For example, papillomas with a high risk for malignant conversion frequently replace K1 ex-

normally restricted to internal epithelia (Nischt et al., 1988; Gimenez-Conti et al., 1990). Regions of premalignant papillomas that express K13 do not normally express K1. To monitor keratin gene expression in *c-fos*-deficient papillomas, we stained serial sections of tumors with antibodies prepared against an array of keratins. Surprisingly, K13 expression was ubiquitous in the suprabasal layers of *c-fos*-deficient papillomas. More significantly, though, while K1 and K13 expression was mutually exclusive in wild-type papillomas, these two keratins were invariably coexpressed in the same cells in the mutant papillomas

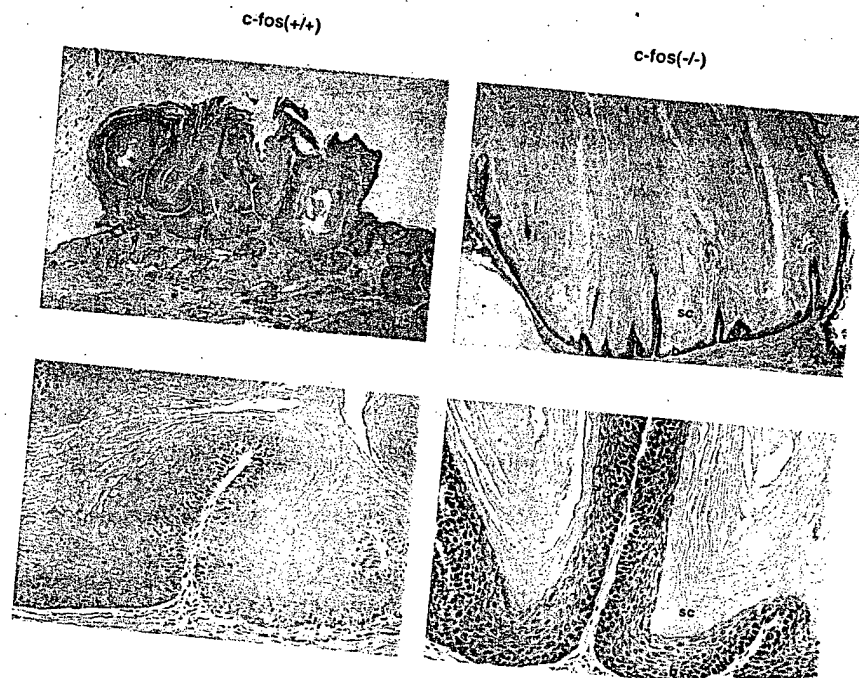


Figure 4. Histology of Papillomas. Hematoxylin and eosin-stained sections of 2-month-old tumors (3 weeks after TPA removal). The top panels show a wild-type and a *c-fos*-deficient tumor at the same level of magnification (13 \times). Note the striking hyperkeratinization of the *fos*-less papilloma and the abrupt transition between the basal (b) and the cornified layers (sc). At higher magnification (135 \times ; same for both genotypes), the contraction of the layers in the *c-fos*-deficient tumor becomes more evident.

c-fos-deficient papillomas examined (Figure 6). Since these type 1 and type 2 keratins do not normally form filament pairs, coexpression of K1 and K13 in the same cells could result in the formation of an irregular intermediate filament network that may contribute to the unusual amount of cornification displayed by mutant papillomas. In contrast with wild-type cornified envelopes, which disassociate when tumors are boiled, large clumps of tightly connected *c-fos*-deficient envelopes endured even after the mutant papillomas were boiled for 5 hr in 2% SDS (data not shown). The considerable durability of *c-fos*-deficient cornified envelopes may reflect interesting underlying biochemical differences, such as covalent modifications in the structure of the envelopes.

c-fos-Deficient Keratinocytes Expressing v-H-ras Do Not Form Tumors When Grafted onto Nude Mice

The development of a malignant neoplasm is a process that involves an intense interaction between tumor cells and normal neighboring cells (reviewed by Paweletz and Boxberger, 1994). The inability of *c-fos*-deficient papillomas to progress into malignant tumors could be due to an intrinsic defect in the v-H-ras-expressing keratinocytes that form the epidermal component of the papillomas. Alternatively, it is possible that malignant tumors do not arise in these animals because the nontransformed *c-fos*-deficient cells neighboring the neoplastic keratinocytes do not provide an environment that is conducive for malignant

ternatively, it is possible that malignant tumors do not arise in these animals because the nontransformed *c-fos*-deficient cells neighboring the neoplastic keratinocytes do not provide an environment that is conducive for malignant

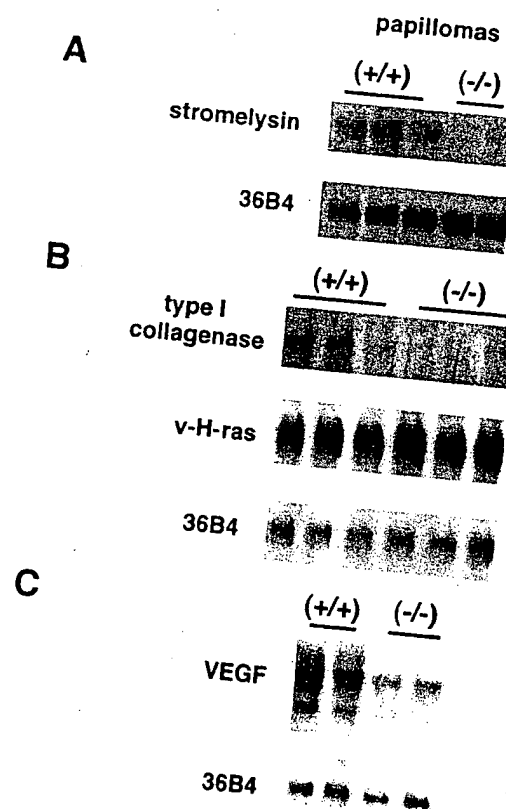


Figure 5. Expression of *c-fos*-Regulated Genes in Papillomas. RNA was prepared from papillomas 1 month after the end of promotion and analyzed by Northern blot. Each lane contains total RNA from a pool of six to eight tumors. Probes are as indicated. Blots were stripped and reprobed.

Table 2. Incidence of Malignant Tumors

Genotype	Malignant Tumors	Percent Affected
<i>c-fos</i> wild-type transgenic v-H-ras	4 of 7	57
<i>c-fos</i> heterozygous transgenic v-H-ras	7 of 17	41
<i>c-fos</i> deficient transgenic v-H-ras	0 of 13	0

Mice were monitored weekly during 45 weeks for the appearance of malignancies. At the end of that time, all mice were sacrificed, and any remaining tumors were harvested for pathological analysis. Individual mice were sacrificed after the first malignant tumor was detected.

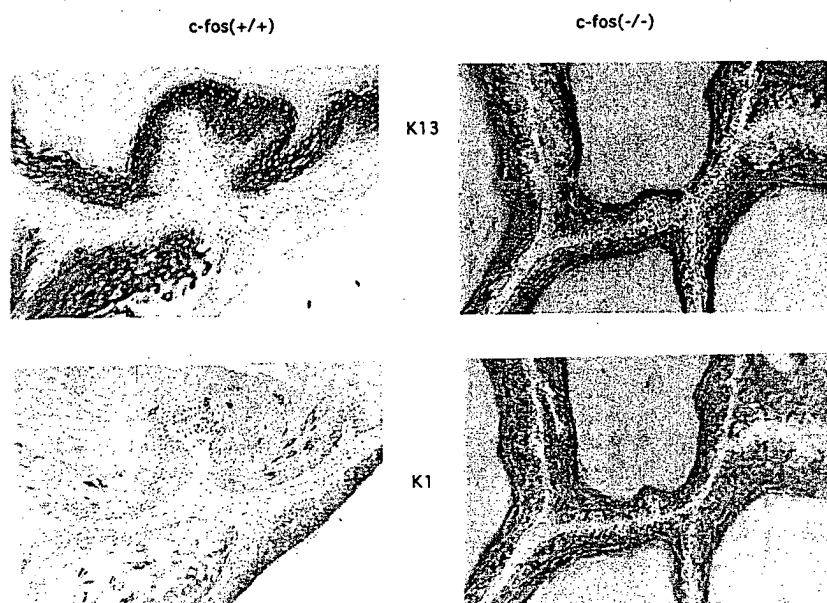


Figure 6. Aberrant Keratin Gene Expression in *c-fos*-Deficient Papillomas

Serial sections of tumors (obtained 2.5 months after the start of the study) were stained with antibodies against K1 (bottom) and K13 (top) and photographed at the same magnification (32 \times). Note the coexpression of K1 and K13 in *c-fos*-deficient tumors.

growth. To establish whether *c-fos*-deficient keratinocytes display a cell-autonomous defect that prevents them from giving rise to malignant tumors, we tested the ability of v-H-ras-expressing mutant keratinocytes to form tumors when grafted onto the back of wild-type hosts (Roop et al., 1986). Primary keratinocytes derived from *c-fos*-deficient, heterozygous, and wild-type mice were infected with a helper-free retrovirus, a variant Harvey murine sarcoma virus (Ha-MSV), expressing an oncogenic form of H-ras (Roop et al., 1986), were mixed with wild-type dermal fibroblasts, and were grafted onto the back of athymic nude mice. Grafted Ha-MSV-infected wild-type keratinocytes gave rise to large papillomas. In contrast, grafts of infected *c-fos*-deficient keratinocytes did not produce significant tumors. Rather, v-H-ras-expressing cells lacking *c-fos* generated a normal epidermis that was hyperplastic in some cases. Interestingly, grafts of keratinocytes heterozygous for the *c-fos* null mutation gave rise to small tumors of variable size that on average were approximately 35% the size of their wild-type counterparts. While there was some variation in absolute tumor volume values, the same trend was observed in three independent experiments: v-H-ras-expressing keratinocytes homozygous for the *c-fos* null mutation failed to produce tumors ($n = 8$), heterozygous cells generated small, fragmented papillomas ($n = 12$), and wild-type keratinocytes gave rise to large tumors ($n = 14$). Mice from a representative experiment are shown in Figure 7A. The presence of black pigment in mice with grafts of *c-fos*-deficient keratinocytes indicates that the graft was successful. Figure 7B displays the evolution of tumor volume as a function of time for that same experiment. Mice with *c-fos*-deficient grafts failed to develop tumors, even when monitored for 3 months (6 weeks beyond the normal observation period). Since tumor formation in this system is dependent upon oncogenic H-ras expression, the levels of mutant Ras protein in keratinocytes used for grafting were examined. All Ha-MSV-

equivalent levels of exogenous Ras in vitro as well as in vivo (data not shown). The growth rate in vitro of all v-H-ras-expressing keratinocytes was similar, regardless of *c-fos* genotype (data not shown). Furthermore, implanted *c-fos* null keratinocytes coinfecting with Ha-MSV and a v-*fes* retrovirus generated dysplastic tumors indistinguishable from those produced by similarly infected wild-type cells, confirming that a *fes*-dependent function is responsible for the deficiency in tumor formation of mutant cells (data not shown). These transplantation experiments illustrate that v-H-ras-expressing keratinocytes derived from *c-fos* null animals appear to have a cell-autonomous defect that hampers tumorigenesis.

Discussion

Tumorigenesis is a complex process that requires alterations in many genes involved in the regulation of cell growth. Many of these genes function outside of the nucleus of the cell and must ultimately exert their effects by communicating with transcription factors. The AP-1 family is believed to be one of the nuclear targets of several signal transduction pathways, in particular the Ras-MAP kinase pathway (reviewed by Hill and Treisman, 1995). Because these pathways are frequently dysregulated by activated oncogenes, it is of considerable interest to determine whether AP-1 factors are actually necessary for cancer development. We have used the well-characterized model of multistep mouse skin carcinogenesis to examine the unfolding of the neoplastic process in the absence of *c-fos*, an important member of the AP-1 family. Our results demonstrate that *c-fos* is required for the development of malignant skin tumors.

The Role of *c-fos* in Initiation and in Promotion

To test the role of *c-fos* in the various steps of skin carcinogenesis, we mated mice heterozygous for the *c-fos* null mu-

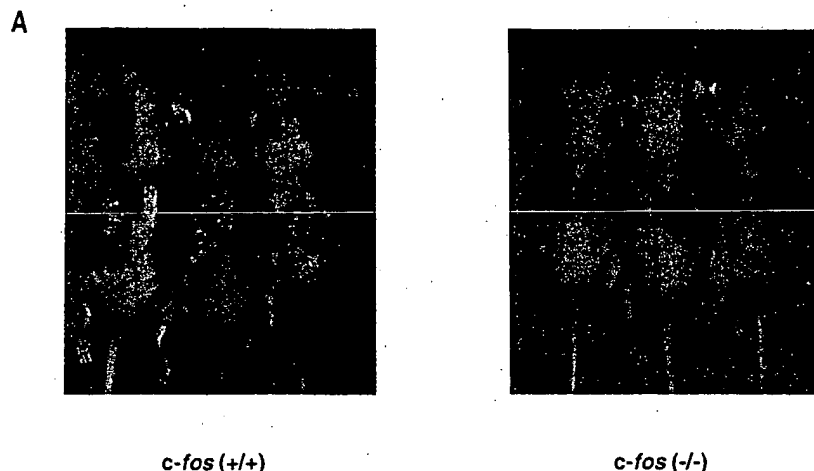
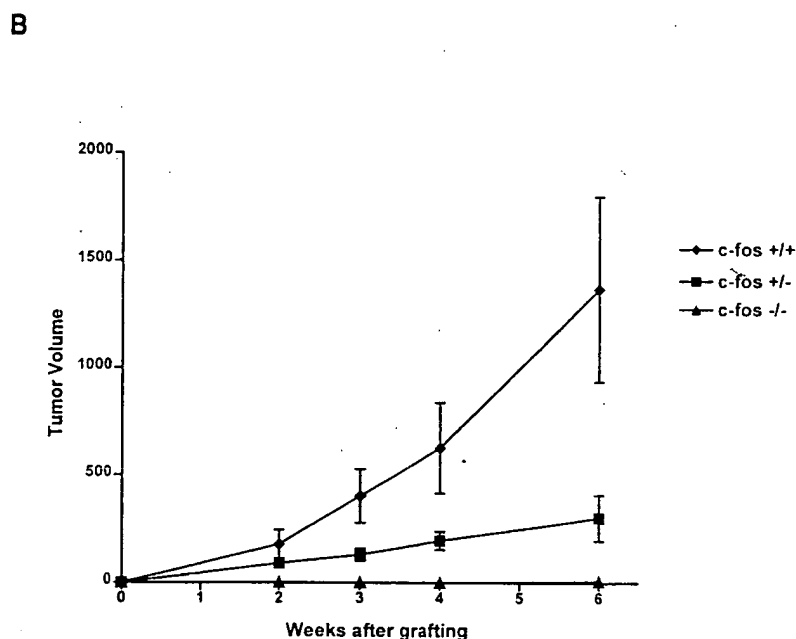


Figure 7. Grafting of v-H-ras-Expressing Keratinocytes

(A) Appearance of grafts of Ha-MSV-infected keratinocytes 6 weeks after implantation. The genotype of grafted keratinocytes is shown below each picture. The presence of pigment (denoting cells of the C57BL/6J \times 129/SvJ genetic background) indicates that grafts were successful.

(B) Tumor development as a function of time for grafts of wild-type ($n = 4$), heterozygous ($n = 5$), and *c-fos*-deficient ($n = 3$) v-H-ras-expressing keratinocytes. Curves represent the average tumor volume for each group. Error bars denote SEM. Note that *c-fos*-deficient cells did not give rise to tumors.



that can substitute for a chemical initiation step. Even though mutations in *H-ras* are very common during initiation in DMBA/TPA protocols, skin tumors induced with other carcinogens frequently do not bear *H-ras* mutations (Bremner et al., 1994). This observation suggests that mutations in other unspecified genes can also serve as initiating events. To examine whether *c-fos* could be one of these unidentified genes, we treated *c-fos* null mice not carrying the v-*H-ras* transgene with the tumor promoter TPA. The absence of papilloma formation established that a *c-fos* null mutation cannot serve as an initiation event.

Mutant mice bearing the v-*H-ras* transgene were able to develop papillomas normally upon TPA treatment. That *c-fos*-deficient papillomas appeared with standard kinetics and in comparable numbers to wild-type tumors shows that, under these experimental conditions, *c-fos* is not required for benign tumor formation. Because *c-fos* is an immediate-early gene whose expression is often used as a marker of cellular proliferation, it may have been anticipated that the main contribution of *c-fos* to oncogenesis would be either to accelerate the rate of cell division or to prevent exit from the cell cycle. Clearly, this does not

seem to be the case in this study. It is possible that during promotion, TPA induces the expression of other genes that can functionally complement the absence of *c-fos*. Nonetheless, since in the vast majority of experimental carcinogenesis protocols, TPA promotion is continued for many weeks, the real relevance, if any, of *c-fos* for tumor promotion would also be concealed under those circumstances. Thus, we can conclude that, while it may be important, *c-fos* is not essential for tumor promotion brought about by TPA treatment.

Abnormalities of Epidermal Differentiation in *c-fos*-Deficient Papillomas

Although initially identical to wild-type papillomas, shortly after the end of TPA treatment, *c-fos*-deficient tumors began to manifest some clear differences. Over time, *c-fos*-deficient papillomas are characterized by a grotesque hyperkeratinization. Mutant papillomas evolve into horny projections covered by a crust of keratin that can grow as long as 2–3 cm. The development of such elongated, hyperkeratinized papillomas (with the great frequency found in

col. It was surprising to find universal K13 expression in these apparently benign tumors, for K13 induction has heretofore been considered a useful diagnostic of premalignant progression. It is important to note that these *c-fos*-mediated differences in epidermal differentiation are only apparent within the context of carcinogenesis, for no differentiation abnormalities were detected in untreated *c-fos*-deficient epidermis. Hence, it is conceivable that the observed differences are contingent on the expression of a mutant H-Ras protein. It is also possible that the expression of the various members of the AP-1 family may be abnormally regulated during tumorigenesis. Given the large number of AP-1 sites that have been identified in epidermal differentiation genes, disorderly expression of other AP-1 members in the absence of *c-fos* may have profound effects. Using immunohistochemistry, we have been unable to recognize significant differences in the expression pattern of AP-1 genes in wild-type and mutant papillomas (data not shown). However, since the activity of AP-1 proteins can be regulated by reversible phosphorylation, it is possible that differences may exist in AP-1-dependent gene expression that are not reflected in an unusual pattern of AP-1 family protein expression.

The Role of *c-fos* in Papilloma Survival and Malignant Progression

In contrast with wild-type and heterozygous tumors, not a single *c-fos*-deficient papilloma progressed to malignancy by the end of the study. Several explanations can potentially account for this observation. It is well established that *c-fos* controls the expression of a variety of genes that are up-regulated during malignant progression, including those encoding the tumor metalloproteases stromelysin and type I collagenase (Kerr et al., 1988; Schonthal et al., 1988; Hennigan et al., 1994). The tumor metalloproteases are a group of secreted enzymes that can degrade the extracellular matrix, thereby facilitating tumor growth, invasion, and metastasis (reviewed by Liotta and Stetler-Stevenson, 1990). Increased metalloprotease expression has been associated with malignant progression in many models of *in vivo* cancer development (reviewed by McDonnell and Matrisian, 1990). Given that the transcripts for stromelysin and type I collagenase were undetectable in RNA obtained from pools of mutant papillomas, it is likely that a protease insufficiency may contribute to the lack of malignant behavior of *c-fos*-deficient tumors.

The sparse external vascularity displayed by *c-fos*-deficient papillomas suggests a second possible factor for the benign nature of *c-fos*-deficient tumors. At a molecular level, the formation of new blood vessels is a process remarkably similar to tumor invasion and metastasis, for angiogenesis also requires cells to traverse normal tissue boundaries and degrade the basement membrane of the tissue to be vascularized (reviewed by Liotta et al., 1991). *c-fos* could be mediating tumor angiogenesis not only through its control of the proteases that are critical for these processes, but also by modulating the expression of the growth factors that induce neovascularization, such

pools of *c-fos*-deficient tumors. Finally, it is conceivable that the abnormal pattern of differentiation of *c-fos*-deficient papillomas may interfere or be incompatible with ordinary tumor progression.

Diminished expression of tumor metalloproteases and angiogenic factors may also contribute to the inability of grafted v-H-ras-expressing *c-fos*-deficient keratinocytes to generate papillomas. The development of an established graft/tumor involves complex fibroblast-keratinocyte interactions that must ultimately result in, among other things, the creation of a new blood vessel network to support the growth of the graft (Smola et al., 1993; Borchers et al., 1994). In the multistep carcinogenesis system, the requirement for angiogenesis may be less stringent, at least for the early stages of tumor development. Since tumors are induced in skin that is already vascularized, initially neoplastic cells need only to expand the existing capillary network, not to establish a completely new one. It is also possible that chemical treatment in the multistep carcinogenesis system induces the expression of genes that allow papilloma formation in the absence of *c-fos*. Many genes, including those encoding the tumor metalloproteases stromelysin and type I collagenase, are known to be expressed in response to tumor promoter stimulation; *c-fos*-deficient fibroblasts can express a limited amount of these proteases when stimulated with TPA (Krieg et al., 1988; Hu et al., 1994). The defect in tumor development observed in the grafting system could also be due to an autocrine overproduction of a growth-inhibiting molecule (e.g., transforming growth factor β) by *c-fos*-deficient cells. Alternatively, v-H-ras-expressing mutant cells may be more sensitive to constraining signals emanating from the wild-type cells that constitute the stromal component of the implant. The experimental flexibility of the skin grafting system should permit the reintroduction of proteases and angiogenic factors to test whether the cell-autonomous defect in tumorigenesis of *c-fos*-deficient keratinocytes is linked to the expression of these genes.

Although the specific details of why *c-fos*-deficient papillomas fail to progress to malignancy are yet to be elucidated, these findings might provide a molecular mechanism for the observation that treatment with retinoids and glucocorticoids can prevent malignant progression of mouse skin tumors, as well as the occurrence of secondary tumors in carcinomas of the head and neck in humans (Hong et al., 1990; Strawhecker and Pelling, 1992; De Luca et al., 1993). Both, the retinoic acid receptors and the glucocorticoid receptor are known to antagonize AP-1-dependent transcription directly (Schule et al., 1990; Kerpola et al., 1993). Thus, it is possible that the inhibitory action of these hormones on AP-1 activity is at least partially responsible for their antitumorigenic effects.

Relevance to Other Neoplastic Processes

An important question is whether these findings will be meaningful for tissues other than the epidermis. We have indirectly addressed this issue by monitoring the frequency of the spontaneous tumors induced by the v-H-ras

nonsolid neoplasms, it appears to be critical for the development of certain solid tumors, such as odontogenic fibrosarcomas (unpublished data). Moreover, a variety of other human and rodent tumors are known to overexpress *c-fos* (e.g., Honoki et al., 1992; Urabe et al., 1992). One of these tumors is human squamous cell carcinoma of the lung (Volm et al., 1992; Wodrich and Volm, 1993). Because the development of this cancer naturally requires repeated applications of a tumor promoter (e.g., tobacco smoke), many consider this human cancer the most accurate reflection of the mouse skin model. Another clinically relevant tumor in which *c-fos* overexpression is associated with malignant progression is human breast cancer (Biunno et al., 1988; Walker and Cowl, 1991).

Our findings highlight the significance of *c-fos* for full neoplastic development. Since malignant progression is the most critical step in terms of host survival, the identification of a gene central to this process represents a promising step toward the therapeutic prevention of neoplastic disease. Furthermore, because *c-fos* is not required for the viability of the whole organism, it may represent an attractive target for pharmacological intervention.

Experimental Procedures

Animals

c-fos-deficient mice were generated as previously described (Johnson et al., 1992). Mice heterozygous for the *c-fos* null mutation (50% 129/SvJ; 50% C57BL/6J) were mated with FvB/N mice homozygous for the TG.AC transgene obtained from Charles River Laboratories. Offspring carrying both the TG.AC transgene and the *c-fos* mutation were identified and interbred. Their progeny was typed for both mutations and divided into the groups shown in Table 1. At 12 weeks of age, mice were shaved, and their dorsal epidermis was treated with either 5 μ g of TPA (Sigma) dissolved in 100 μ l of acetone or acetone alone. To normalize for their reduced size (and hence smaller dorsal surface area), *c-fos*-deficient mice received 55% of the wild-type dose of promoter. This dose of TPA resulted in a similar papilloma yield in wild-type and mutant mice (see Table 1). Treatment was applied twice a week for 5 weeks. Male mice were caged individually to prevent wound-induced tumors. All mice were examined at least once a week for a period of 45 weeks.

Histology and Immunohistochemistry

For pathological analysis, complete autopsies were performed and tissues were fixed in Optifix. Tumors were not removed from mice under observation. For fluorescent immunohistochemistry, neonatal skin was frozen in OCT (Miles Incorporated). Double-labeled indirect immunofluorescence was performed as described by Nischt et al. (1988). Antisera against mouse keratins and loricrin are characterized by Yuspa et al. (1989) and Mehrel et al. (1990).

Cell Culture and Retroviral Infections

Breeding pairs of animals heterozygous for the *c-fos* null mutation were monitored daily for the appearance of offspring. Neonatal mice were genotyped by PCR (Johnson et al., 1992). After sacrifice, animals were kept on ice until typing results became available (24 hr). The skin from selected mice was removed, and primary keratinocytes were prepared as described by Hennings et al. (1980). Dermal fibroblasts from the same *c-fos* wild-type mice used to derive keratinocytes to be grafted were cultured separately for 1 week (Yuspa et al., 1976). Primary keratinocytes were infected 3 days after plating at a multiplicity of 1 with a replication-defective variant of Ha-MSV devoid of helper virus and containing the *v-H-ras* gene (Roop et al., 1986). After 5 days, keratinocytes and fibroblasts were combined and grafted. Wild-type fibroblasts were used for grafting keratinocytes of all three genotypes. Successful Ha-MSV infection and p21 H-Ras expression were monitored by analyzing total protein extracts of infected keratinocytes by

Western blot and by immunohistochemistry on grafts, using an antibody against p21 H-Ras (Transduction Laboratories).

In Vivo Grafting

Combined cell pellets were transplanted onto 8- to 12-week-old athymic nude mice as described by Strickland et al. (1993). The volume of tumors was measured on a weekly basis, starting 2 weeks after grafting. Mice were sacrificed after 6 weeks of observation, unless otherwise noted.

Statistical Analysis

Statistical work was performed by the Division of Biostatistics at the Dana-Farber Cancer Institute. The associations between *c-fos* genotype and the incidence of epidermal tumors were analyzed with log linear models using GLIM (GLIM4, 1992 Royal Statistical Society, London), evaluating the association both assuming and not assuming and ordering to the genotype (+/+ > +/- > -/-); *p* values of <0.05 were considered significant.

Acknowledgments

Correspondence should be addressed to B. M. S. We thank Aya Leder and Raymond Tennant for advice regarding use of the TG.AC strain, Randall Johnson for suggestions regarding the care and genotyping of *c-fos* mutant mice, Andrzej Dlugosz and Ulrike Lichti for helpful discussions, Adam Glick for the *v-fos* retrovirus, Robert Cardiff and Robert Munn for histopathology and photography of tumors, Rodrigo Bravo for antibodies against AP-1 proteins, and Janet Andersen for statistical analysis. This work was supported by grants from the National Institutes of Health (HD27295) and from the Sandoz/Dana-Farber Cancer Institute Drug Discovery Program.

Received March 13, 1995; revised July 7, 1995.

References

- Angel, P., and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* 1072, 129-157.
- Binetruy, B., Smeal, T., and Karin, M. (1991). Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature* 351, 122-127.
- Biunno, I., Pozzi, M. R., Pierotti, M. A., Pilotti, S., Cattoretti, G., and Della, P. G. (1988). Structure and expression of oncogenes in surgical specimens of human breast carcinomas. *Br. J. Cancer* 57, 464-468.
- Borchers, A. H., Powell, M. B., Fusenig, N. E., and Bowden, G. T. (1994). Paracrine factor and cell-cell contact-mediated induction of protease and *c-ets* gene expression in malignant keratinocyte/dermal fibroblast cocultures. *Exp. Cell Res.* 213, 143-147.
- Bremner, R., Kemp, C. J., and Balmain, A. (1994). Induction of different genetic changes by different classes of chemical carcinogens during progression of mouse skin tumors. *Mol. Carcinog.* 11, 90-97.
- Buchmann, A., Ruggeri, B., Klein, S. A., and Balmain, A. (1991). Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-ras alleles on chromosome 7. *Cancer Res.* 51, 4097-4101.
- Curran, T., Peters, G., Van, B. C., Teich, N. M., and Verma, I. M. (1982). FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* 44, 674-682.
- De Luca, L. M., Sly, L., Jones, C. S., and Chen, L.-C. (1993). Effects of dietary retinoic acid on skin papilloma and carcinoma formation in female SENCAR mice. *Carcinogenesis* 14, 539-542.
- Deng, T., and Karin, M. (1994). c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. *Nature* 371, 171-175.
- DiGiovanni, J. (1992). Multistage carcinogenesis in mouse skin. *Pharmacol. Therapeut.* 54, 63-128.
- Fisher, C., Byers, M. R., Iadarola, M. J., and Powers, E. A. (1991). Patterns of epithelial expression of Fos protein suggest important role

- in the transition from viable to cornified cell during keratinization. *Development* 111, 253-258.
- French, J. E., Libbus, B. L., Hansen, L., Spalding, J., Tice, R. R., Mahler, J., and Tennant, R. W. (1994). Cytogenetic analysis of malignant skin tumors induced in chemically treated TG.AC transgenic mice. *Mol. Carcinog.* 11, 215-226.
- Gimenez-Conti, I., Aldaz, C. M., Bianchi, A. B., Roop, D. R., Slaga, T. J., and Conti, C. J. (1990). Early expression of type I K13 keratin in the progression of mouse skin papillomas. *Carcinogenesis* 11, 1995-1999.
- Hansen, L. A., and Tennant, R. (1994a). Focal transgene expression associated with papilloma development in v-Ha-ras-transgenic TG.AC mice. *Mol. Carcinog.* 9, 143-154.
- Hansen, L. A., and Tennant, R. W. (1994b). Follicular origin of epidermal papillomas in v-Ha-ras transgenic TG.AC mouse skin. *Proc. Natl. Acad. Sci. USA* 91, 7822-7826.
- Hennigan, R. F., Hawker, K. L., and Ozanne, B. W. (1994). Fos-transformation activates genes associated with invasion. *Oncogene* 9, 3591-3600.
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19, 245-254.
- Hennings, H., Glick, A. B., Greenhalgh, D. A., Morgan, D. L., Strickland, J. E., Tennenbaum, T., and Yuspa, S. H. (1993). Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. *Proc. Soc. Exp. Biol. Med.* 202, 1-8.
- Hilberg, F., Aguzzi, A., Howells, N., and Wagner, E. F. (1993). *c-jun* is essential for normal mouse development and hepatogenesis. *Nature* 365, 179-181.
- Hill, C. S., and Treisman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 80, 199-211.
- Hong, W. K., Lippman, S. M., Itri, L. M., Karp, D. D., Lee, J. S., Byers, R. M., Schantz, S. P., Kraemer, A. M., Lotan, R., Peters, L. J., Dimery, I. W., Brown, B. W., and Goepfert, H. (1990). Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* 323, 795-801.
- Honoki, K., Tsutsumi, M., Tsujiuchi, T., Kondoh, S., Shiraiwa, K., Miyauchi, Y., Mii, Y., Tamai, S., Konishi, Y., and Bowden, G. T. (1992). Expression of the transin, *c-fos*, and *c-jun* genes in rat transplantable osteosarcomas and malignant fibrous histiocytomas. *Mol. Carcinog.* 6, 122-128.
- Hu, E., Mueller, E., Oliviero, S., Papaioannou, V. E., Johnson, R., and Spiegelman, B. M. (1994). Targeted disruption of the *c-fos* gene demonstrates *c-fos*-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes. *EMBO J.* 13, 3094-3103.
- Johnson, R. S., Spiegelman, B. M., and Papaioannou, V. (1992). Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene. *Cell* 71, 577-586.
- Johnson, R. S., Van, L. B., Papaioannou, V. E., and Spiegelman, B. M. (1993). A null mutation at the *c-jun* locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* 7, 1309-1317.
- Kerppola, T. K., Luk, D., and Curran, T. (1993). Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity *in vitro*. *Mol. Cell. Biol.* 13, 3782-3791.
- Kerr, L. D., Holt, J. T., and Matrisian, L. M. (1988). Growth factors regulate transin gene expression through *c-fos* dependent and *c-fos* independent pathways. *Science* 242, 1424-1428.
- Klagsbrun, M., and Soker, S. (1993). VEGF/VPF: the angiogenesis factor found? *Curr. Biol.* 3, 699-702.
- Klein-Szanto, A. J. P., Larcher, F., Bonfil, R. D., and Conti, C. J. (1989). Multistage chemical carcinogenesis protocols produce spindle cell carcinomas of the mouse skin. *Carcinogenesis* 10, 2169-2172.
- Krieg, P., Finch, J., Fustenberg, G., Melber, K., Matrisian, L. M., and Bowden, G. T. (1988). Tumor promoters induce a transient expression of tumor-associated genes in both basal and differentiated cells of the mouse epidermis. *Carcinogenesis* 9, 95-100.
- ras transgene abrogates the initiation step in mouse skin tumorigenesis: effects of phorbol esters and retinoic acid. *Proc. Natl. Acad. Sci. USA* 87, 9178-9182.
- Liotta, L. A., and Stetler-Stevenson, W. G. (1990). Metalloproteinases and cancer invasion. *Semin. Cancer Biol.* 1, 99-106.
- Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 64, 327-336.
- Maki, Y., Bos, T. J., Davis, C., Starbuck, M., and Vogt, P. K. (1987). Avian sarcoma virus 17 carries the jun oncogene. *Proc. Natl. Acad. Sci. USA* 84, 2848-2852.
- Matrisian, L., Bowden, G. T., Krieg, P., Furstenberger, G., Briand, J.-P., Leroy, P., and Breathnach, R. (1986). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. *Proc. Natl. Acad. Sci. USA* 83, 9413-9417.
- McDonnell, S., and Matrisian, L. M. (1990). Stromelysin in tumor progression and metastasis. *Cancer Metastasis Rev.* 9, 305-319.
- Mehrel, T., Hohl, D., Rothnagel, J. A., Longley, M. A., Bundman, D., Cheng, C., Lichti, U., Bisher, M. E., Steven, A. C., Steinert, P. M., Yuspa, S. H., and Roop, D. R. (1990). Identification of a major keratinocyte cell envelope protein, loricrin. *Cell* 61, 1103-1112.
- Nischt, R., Roop, D. R., Mehrel, T., Yuspa, S. H., Rentrop, M., Winter, H., and Schweizer, J. (1988). Aberrant expression during two-stage mouse skin carcinogenesis of a type I 47-kDa keratin, K13, normally associated with terminal differentiation of internal stratified epithelia. *Mol. Carcinog.* 1, 96-108.
- Pawletz, N., and Boxberger, H.-J. (1994). Defined tumor cell-host interactions are necessary for malignant growth. *Crit. Rev. Oncog.* 5, 69-105.
- Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991). Phosphorylation of *c-jun* mediated by MAP kinases. *Nature* 353, 670-674.
- Ransone, L. J., and Verma, I. M. (1990). Nuclear proto-oncogenes *fos* and *jun*. *Annu. Rev. Cell Biol.* 6, 539-557.
- Roop, D. R., Lowy, D. R., Tambourin, P. E., Strickland, J., Harper, J. R., Balaschak, M., Spangler, E. F., and Yuspa, S. H. (1986). An activated Harvey ras oncogene produces benign tumours on mouse epidermal tissue. *Nature* 323, 822-824.
- Ruther, U., Garber, C., Komitowski, D., Muller, R., and Wagner, E. F. (1987). Deregulated *c-fos* expression interferes with normal bone development in transgenic mice. *Nature* 325, 412-416.
- Ruther, U., Komitowski, D., Schubert, F. R., and Wagner, E. F. (1989). *c-fos* expression induces bone tumors in transgenic mice. *Oncogene* 4, 861-865.
- Schonthal, A., Herrlich, P., Rahmsdorf, H. J., and Ponta, H. (1988). Requirement for *fos* gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell* 54, 325-334.
- Schuh, A. C., Keating, S. J., Monteclaro, F. S., Vogt, P. K., and Breitman, M. L. (1990). Obligatory wounding requirement for tumorigenesis in v-jun transgenic mice. *Nature* 346, 756-760.
- Schule, R., Umesono, K., Mangelsdorf, D. J., Bolado, J., Pike, J. W., and Evans, R. M. (1990). Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell* 61, 497-504.
- Smeyne, R. J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T., and Morgan, J. I. (1992). *fos-lacZ* transgenic mice: mapping sites of gene induction in the central nervous system. *Neuron* 8, 13-23.
- Smola, H., Thiekotter, G., and Fusenig, N. E. (1993). Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J. Cell Biol.* 122, 417-429.
- Spalding, J. W., Momma, J., Elwell, M. R., and Tennant, R. W. (1993). Chemically induced skin carcinogenesis in a transgenic mouse line (TG.AC) carrying a v-Ha-ras gene. *Carcinogenesis* 14, 1335-1341.
- Strawhecker, J. M., and Pelling, J. C. (1992). Inhibition of mouse skin tumorigenesis by dexamethasone occurs through a Ha-ras-dependent

- Strickland, J. E., Dlugosz, A. A., Hennings, H., and Yuspa, S. H. (1993). Inhibition of tumor formation from grafted murine papilloma cells by treatment of grafts with staurosporine, an inducer of squamous differentiation. *Carcinogenesis* 14, 205-209.
- Urabe, A., Nakayama, J., Taniguchi, S., Terao, H., and Hori, Y. (1992). Expression of the *c-fos* oncogene in chemically-induced mouse tumours and in human skin tumours. *J. Pathol.* 168, 281-286.
- Volm, M., Efferth, T., Mattern, J., and Wodrich, W. (1992). Overexpression of *c-fos*- and *c-erbB-1* encoded proteins in squamous cell carcinomas of the lung of smokers. *Int. J. Oncol.* 1, 69-71.
- Walker, R. A., and Cowl, J. (1991). The expression of *c-fos* protein in human breast. *J. Pathol.* 163, 323-327.
- Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Mohle, S. U., Ruther, U., and Wagner, E. F. (1992). Bone and haematopoietic defects in mice lacking *c-fos*. *Nature* 360, 741-745.
- Wodrich, W., and Volm, M. (1993). Overexpression of onco-proteins in non-small cell lung carcinomas of smokers. *Carcinogenesis* 14, 1121-1124.
- Yuspa, S. H. (1994). The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. *Cancer Res.* 54, 1178-1189.
- Yuspa, S. H., Ben, T., Patterson, E., Michael, D., Elgjo, K., and Hennings, H. (1976). Stimulated DNA synthesis in mouse epidermal cell cultures treated with 12-O-tetradecanoyl-phorbol-13-acetate. *Cancer Res.* 36, 4062-4068.
- Yuspa, S. H., Kilkenny, A. E., Steinert, P. M., and Roop, D. R. (1989). Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations *in vitro*. *J. Cell Biol.* 109, 1207-1217.

c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo* and *in vitro*

LUCIA MARCONCINI*, SERENA MARCHIOT†, LUCIA MORBIDELLI‡, ELENA CARTOCCHI*, ADRIANA ALBINI§, MARINA ZICHE‡, FEDERICO BUSSOLINO†, AND SALVATORE OLIVIERO*¶

*Dipartimento di Biologia Molecolare Università degli Studi di Siena, via Fiorentina 1–53100 Siena, Italy; †Dipartimento di Genetica, Biologia e Chimica Medica, Università degli Studi di Torino, and Institute for Cancer Research and Treatment, 10060 Candiolì, Italy; ‡Dipartimento di Farmacologia Università degli Studi di Firenze e Istituto di Scienze Farmacologiche Università degli Studi di Siena, 53100 Siena, Italy; and §Istituto Nazionale per la Ricerca sul Cancro e Centro per le Biotecnologie Avanzate, 16100 Genova, Italy

Communicated by Lewis T. Williams, Chiron Technologies, Emeryville, CA, June 18, 1999 (received for review March 4, 1999)

ABSTRACT *c-fos*-induced growth factor/vascular endothelial growth factor D (Figf/Vegf-D) is a secreted factor of the VEGF family that binds to the vessel and lymphatic receptors VEGFR-2 and VEGFR-3. Here we report that Figf/Vegf-D is a potent angiogenic factor in rabbit cornea *in vivo* in a dose-dependent manner. *In vitro* Figf/Vegf-D induces tyrosine phosphorylation of VEGFR-2 and VEGFR-3 in primary human umbilical cord vein endothelial cells (HUVECs) and in an immortal cell line derived from Kaposi's sarcoma lesion (KS-IMM). The treatment of HUVECs with Figf/Vegf-D induces dose-dependent cell growth. Figf/VEGF-D also induces HUVEC elongation and branching to form an extensive network of capillary-like cords in three-dimensional matrix. In KS-IMM cells Figf/Vegf-D treatment results in dose-dependent mitogenic and motogenic activities. Taken together with the previous observations that Figf/Vegf-D expression is under the control of the nuclear oncogene *c-fos*, our data uncover a link between a nuclear oncogene and angiogenesis, suggesting that Figf/Vegf-D may play a critical role in tumor cell growth and invasion.

During development and in the vascularization of tumors inductive signaling leads to the formation of capillaries throughout the new-forming tissues (1, 2). Inhibitors who regulate proliferation, migration, differentiation of endothelial cells, degradation of the extracellular matrix, and tube formation finely tune this complex process, known as angiogenesis (3–5). The prototype of angiogenic factors is represented by vascular endothelial growth factor (VEGF) A, also known as vascular permeability factor. VEGF-A induces endothelial cell differentiation and is essential for embryonic vessel development (4, 6, 7). It belongs to a multigene family of angiogenic factors in which several new members were discovered recently. It generally is thought that each member of this family plays a specific role in the angiogenic process (2). In addition to VEGF-A, this family includes the placental growth factor (PlGF), VEGF-B/VRP, VEGF-C/VRP, *c-fos*-induced growth factor (Figf)/VEGF-D, and VEGF-E (8–18). All of these factors show a conserved cysteine-rich domain characteristic of the family. Differences in the patterns of expression suggest a specific role for at least some of the factors in the vascularization of different tissues. Plgf is expressed mostly in the placenta, whereas VEGF-B is prevalent in skeletal and cardiac muscle tissues (12, 19). These two factors can form heterodimers with VEGF-A adding an additional level of specificity (12, 20). Interestingly, VEGF-C is involved in both blood and lymphatic vessel growth (21, 22).

Figf/Vegf-D initially was identified by using a differential screening strategy aimed at the identification of new *c-fos*-responsive genes in mouse fibroblasts and therefore named *c-fos*-induced growth factor (15). Its human orthologue shares 84% identity and was named VEGF-D because it encodes for a secreted protein whose primary sequence is most similar to VEGF-C (16, 23–25). Both VEGF-C and VEGF-D are recognized by VEGF receptors (VEGFR)-2 and -3, which are present on endothelial cells (14, 23). In mouse embryos Figf/Vegf-D is expressed in several organs, including limb buds, teeth, heart, and pituitary as well as lung and kidney mesenchyme, liver, derma, and periosteum of the vertebral column that partially overlaps Vegf-C expression (26, 27). In cultured fibroblasts Figf/Vegf-D regulation differs from VEGF-C. Whereas the expression of Figf/Vegf-D depends on *c-fos* (15), VEGF-C is induced by serum, tumor promoter phorbol myristate 13-acetate, IL-1 β , and tumor necrosis factor α , and its expression is independent from *c-fos* (15, 28, 29).

We produced a recombinant form of mature mouse Figf/Vegf-D and analyzed its biological activity both *in vivo* and *in vitro*. Figf/Vegf-D, expressed in Chinese hamster ovary (CHO) cells or purified from yeast, is a potent angiogenic factor in rabbit cornea assays. *In vitro* it activates tyrosine phosphorylation of VEGFR-2 and VEGFR-3 present on human umbilical cord vein endothelial cells (HUVECs) and on the Kaposi's sarcoma immortalized cell line (KS-IMM). In KS-IMM cells Figf/Vegf-D induces proliferation and chemotaxis. In HUVECs Figf/Vegf-D induces growth and morphological changes within a three-dimensional matrix.

MATERIALS AND METHODS

Expression of Figf/Vegf-D. To express the mature factor in CHO cells, the Figf/Vegf-D cDNA with a segment coding for the FLAG octapeptide (IBI/Kodak) at C terminal was amplified by PCR and inserted into the mammalian expression vector pcDNA3 (Invitrogen) under the control of the cytomegalovirus promoter (construct LM357). CHO cells were transfected with LM357 by using calcium phosphate precipitation. Stable clones were selected in DMEM containing 10% FCS and 800 μ g/ml G418. To assay the presence of Figf/Vegf-D in CHO supernatants, isolated clones were grown in DMEM containing 2% FCS and 800 μ g/ml G418 and analyzed by ELISA using anti-Figf/Vegf-D rabbit polyclonal antiserum (15). Supernatant from positive clones was precipitated with deoxycholate acid and analyzed by Western blot. Different CHO clones expressed different Figf/Vegf-D levels. Specifi-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: Figf, *c-fos*-induced growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical cord vein endothelial cell; KS-IMM, Kaposi's sarcoma-immortal; CHO, Chinese hamster ovary.

¶To whom reprint requests should be addressed. E-mail: oliviero@unisi.it.

cally, clone 65 expressed less than 0.1 ng/ml of Figf/Vegf-D in the cell supernatant *in vitro* whereas clone 79 expressed approximately 0.5 ng/ml of Figf/VEGF-D in the same conditions.

To express Figf/Vegf-D in yeast a cDNA fragment encoding the portion of the mouse Figf/Vegf-D polypeptide from residues 91 to 208 with six histidine residues at N terminus was amplified by PCR and inserted into the expression vector Yepsec1 immediately downstream from DNA sequence encoding the *Kluyveromyces lactis* toxin leader peptide (LM375) (30). The protein was expressed in *Saccharomyces cerevisiae* yeast strain by adding galactose to the yeast culture medium because Yepsec1 construct contains a galactose upstream activation sequence and the 5' nontranslated leader of the yeast CYC1 gene, up to position -4 from the ATG translation initiation codon (30). Figf/Vegf-D glycosylation mutant was obtained by PCR with the substitution N160P (LM376). Figf/Vegf-D and Figf/Vegf-D N160P proteins were purified from the yeast supernatant by using a nickel column (HiTrap Chelating columns Pharmacia Biotech) under native conditions.

In Vivo Angiogenic Assay. The angiogenic activity of Figf/Vegf-D was assayed *in vivo* by using the rabbit cornea assay previously described (31). Corneal assays were performed in male New Zealand albino rabbits (Charles River, Calco, Lecco, Italy) in accordance with the guideline of the European Economic Community for Animal Care and Welfare (EEC Law No. 86/609). Briefly, after being anaesthetized with sodium pentotal (30 mg/Kg), a micro pocket (1.5 × 3 mm) was surgically produced by using a pliable iris spatula 1.5 mm wide in the lower half of the cornea. The cell suspension (from 2.5 to 4 × 10⁵ cells/5 ml) or slow-release pellets of Elvax-40 (DuPont) containing the purified growth factor were implanted into the micro pocket. Subsequently daily observation of the implants was made with a slit lamp stereomicroscope without anesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 4 days and capillaries progressed to reach the implanted pellet according to the scheme previously reported (32). The potency of angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries, and an angiogenesis score was calculated as described (32). Corneas were removed at the end of the experiment as well as at defined intervals after surgery and/or treatment and fixed in formalin for histological examination. A minimum of four independent experiments was performed for each condition.

Cell Cultures. Human endothelial cells were isolated from umbilical cord vein by collagenase treatment as described (33) and used at passage 1–4. KS-IMM cells were derived from a non-AIDS patient and are immortalized without signs of senescence after more than 120 *in vitro* passages. This cell line shares common markers and similar biological behavior with typical KS "spindle cells" (34). Cells were grown on gelatin-coated plastic, in medium M 199 supplemented with 20% heat-inactivated FCS, penicillin (100 units/ml), streptomycin (50 µg/ml), heparin (50 µg/ml), and bovine brain extract (100 µg/ml) (Life Technologies, Milan, Italy).

In Vitro Angiogenesis. Because Matrigel can induce spontaneously *in vitro* angiogenesis, we tested more preparations and used batches devoid of this activity. Fifty microliters of Matrigel (Collaborative Research, lot 901448) (35) was added per well of 96-well tissue culture plates and allowed to gel at 37°C for 10 min. HUVECs were starved for 24 h in M199 with 1% FCS before being harvested in PBS-EDTA. Cells (10⁴) were gently added to each of triplicate wells and allowed to adhere to the gel coating for 30 min at 37°C. Then, medium was replaced with indicated concentrations of Figf/Vegf-D. The plates were monitored after 24 h and photographed with a Canon microscope. Each experiment was repeated at least three times with identical results.

Immunoprecipitation and Western Blotting. Subconfluent cultures were starved as above and then cells were stimulated with the indicated concentrations of Figf/Vegf-D for 10 min at room temperature. Positive control was done by incubating cells with sodium orthovanadate (0.1 mM H₂O₂, 1 mM Na₃VO₄) for 20 min at 37°C. After three washes with cold PBS containing 1 mM sodium orthovanadate, cells were lysed for 20 min on ice in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na₃VO₄, 1 mM PMSF, 0.1 mM ZnCl₂, 1% Triton. Lysates (1 mg of total proteins) were incubated at 4°C for 2 h with 100 µl of a 50% solution of protein A-Sepharose (Amersham-Pharmacia Biotech) in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and anti-VEGFR-2 (Santa Cruz Biotechnology, sc-504) or anti-VEGFR-3 (Santa Cruz Biotechnology, sc-321). Immunoprecipitates were washed four times with lysis buffer and analyzed by 8% SDS/PAGE. Proteins were transferred onto a nylon membrane [poly(vinylidene difluoride), Millipore] and analyzed by immunoblotting with antiphosphotyrosine mAb (Upstate Biotechnology, Lake Placid, NY). Staining was performed by a chemiluminescence assay (ECL, Amersham-Pharmacia Biotech).

Cell Growth Assay. HUVECs (2.5 × 10³) or KS-IMM cells were plated in 96-well plates (Costar) coated with gelatin (Difco; 0.05%, for 1 h at 22°C) in M199 medium containing 20% FCS (Irvine Scientific). After 24 h the medium was removed and replaced with M199 containing 1% FCS with or without Figf/Vegf-D; fresh factor was added every 2 days. Endothelial cell numbers were estimated after staining with crystal violet by a colorimetric assay described by Keung *et al.* (36).

Chemotaxis Assay. Chemotaxis assays on HUVECs and KS-IMM were performed as described (33, 37) with the Boyden chamber technique using a 48-well micro chemotaxis chamber. Polyvinylpyrrolidone-free polycarbonate filters (Nucleopore, Corning-Costar) with a pore size of 5 µm were coated with 1% gelatin for 10 min at room temperature and equilibrated in M199 supplemented with 1% FCS. Indicated concentrations of purified Figf/Vegf-D were placed in the lower compartment of a Boyden chamber. Subconfluent cultures were starved as above, harvested in PBS (pH 7.4) with 10 mM EDTA, washed once in PBS, and resuspended in M199 containing 1% FCS, at a final concentration of 2.5 × 10⁶ cells/ml. After placing the filter between the lower and upper chambers, 50 µl of the cell suspension was seeded in the upper compartment. Cells were allowed to migrate for 7 h at 37°C in a humidified atmosphere with 5% CO₂. The filter then was removed, and cells on the upper side were scraped with a rubber policeman. Migrated cells were fixed in methanol, stained with Giemsa solution (Diff-Quick, Baxter Diagnostics, Rome) and counted from five random high-power fields (magnification ×100) in each well. Each experimental point was studied in triplicate.

RESULTS

Induction of Angiogenesis *in Vivo*. Mature VEGF-C and Figf/VEGF-D factors are generated by proteolytic cleavages of both of the N- and C-terminal domains during secretion (15, 23, 38). To obtain recombinant mature Figf/Vegf-D we generated CHO clones by stable transfection of constructs containing the mouse Figf/Vegf-D cDNA truncated at the C-terminal proteolytic site (38). To assess *in vivo* the angiogenic activity of increasing concentrations of the recombinant protein administered to avascular tissue two clones expressing different levels of secreted Figf/Vegf-D were selected for implantation into rabbit corneas. These clones secrete in the culture medium Figf/Vegf-D in two main forms of molecular mass of 30 and 21 kDa, respectively (Fig. 14). Both clone 65 and clone 79 induced corneal vascularization whereas the CHO mock transfectant clone did not show any angiogenic

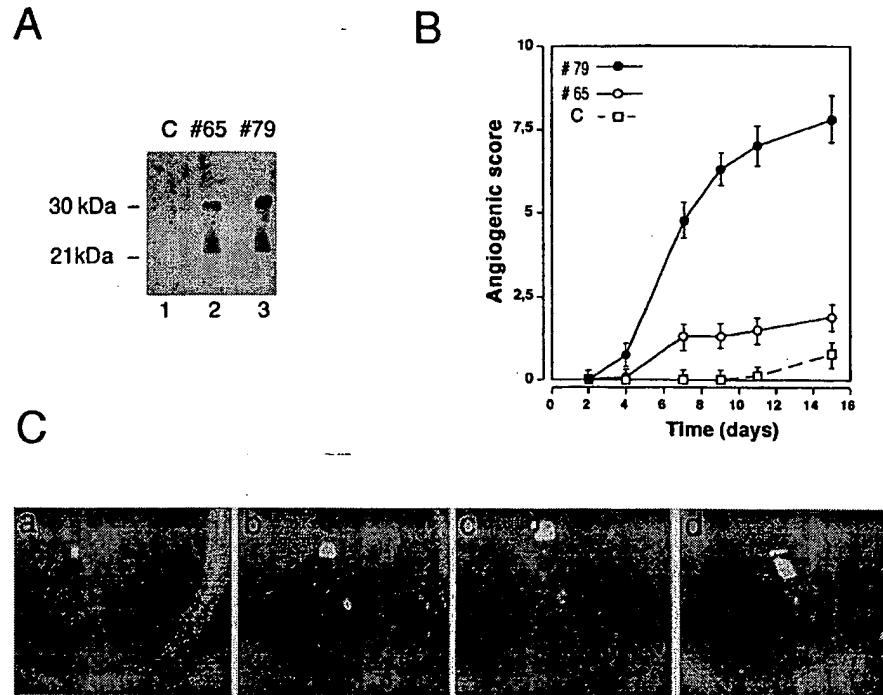


FIG. 1. Implanted Figf/Vegf-D-expressing cells induce neovascularization in rabbit corneas. (A) Figf/Vegf-D expressed in CHO cells. Equal volumes of culture supernatants from clones 65 and 79 were precipitated and analyzed by Western blot using an anti-Figf/Vegf-D rabbit polyclonal antiserum. (B) CHO cells (4×10^4) expressing Figf/Vegf-D were surgically implanted into the corneas. New blood vessel growth was recorded every other day with a slit lamp stereomicroscope. Angiogenic scores were calculated on the basis of the number of vessels and their growth rate and plotted versus time (for experimental details see *Materials and Methods*). Angiogenic score data are the mean values obtained from the response scored in all animals in this study. C, CHO mock transfectant clone; #65, clone expressing low levels of Figf/Vegf-D (0.1 ng/ml protein in supernatant); #79 clone expressing higher levels of Figf/Vegf-D (approximately 0.5 ng/ml protein in supernatant). (C) Pictures of rabbit corneas from a representative experiment. (a) Corneal implant of CHO mock transfectant. Clone 79 promotes and sustains vascular growth over time at day 6 (b), 9 (c), and 14 (d). Corneas were photographed with a stereomicroscope. Magnification: $\times 18$.

effect (Fig. 1B). Although a direct dose response could not be made in this assay, the efficiency of the angiogenic response correlated with the amount of growth factor released *in vitro* as clone 79 secreted about 5-fold more Figf/Vegf-D than clone 65 in the same conditions (Fig. 1A). Consistently, neovascular growth induced by clone 79 was more efficient and persisted in 100% of the implants whereas clone 65 did so in only 30% of corneas (Fig. 1B). This angiogenic activity also was suggested by the direct correlation between neovascular growth observed and the number of cells implanted into corneal micro pocket (data not shown). The angiogenic response obtained with clone 79 (Fig. 1C) was comparable to the one elicited with cells expressing VEGF-A₁₂₁ (39) both in intensity and appearance.

To obtain larger amounts of pure Figf/Vegf-D it also was expressed in yeast *S. cerevisiae*. To obtain a secreted Figf/Vegf-D form in yeast supernatants the cDNA fragment encoding the portion of the mouse Figf/Vegf-D polypeptide from residues 91 to 208 plus a segment coding for six histidine residues at the N terminus was cloned in a yeast vector containing a secretion signal. This recombinant protein expressed in yeast was secreted into the culture medium (Fig. 2A). By contrast with the other members of the VEGF family, VEGF-C and Figf/Vegf-D contain two putative glycosylation sites in the mature protein. Secreted Figf/Vegf-D is glycosylated at asparagine-160 residue in both mammalian and yeast cells (data not shown). To test the activity of both the glycosylated and unglycosylated forms we also generated a Figf/Vegf-D mutant in which the glycosylation site was mutated by the introduction of a proline residue at position 160, which is present in all other known VEGF family members. Consistent with N-linked glycosylation, the wild-type protein shows about 2-kDa molecular mass increase with respect to the

mutant Figf/Vegf-D N160P (Fig. 2A) and it is sensitive to endoglycosidase H (not shown).

Figf/Vegf-D purified to homogeneity was analyzed in the corneal micro pocket assay *in vivo*. Similar to the results obtained with implanted CHO cells, purified Figf/Vegf-D induced a strong angiogenic response. After the implant of a single dose of protein in the slow-release pellets all Figf/Vegf-D doses of 100–400 ng/pellet induced capillary growth after just 3 days. However, a clear effect of increasing Figf/Vegf-D concentration was evident at later time points (Fig. 2B). The Figf/Vegf-D N160P mutant showed less potent angiogenic activity with respect to the wild-type protein (Fig. 2C), suggesting that Figf/Vegf-D glycosylation is involved in receptor recognition. In this assay, recombinant Figf/Vegf-D showed intermediate activity when compared with VEGF-A₁₂₁ and VEGF-A₁₆₅ (Fig. 2D) when used at doses of 300–400 ng. Corneal angiogenesis induced by either Figf/Vegf-D or VEGF-A was noninflammatory (not shown).

Figf/Vegf-D Induces *in Vitro* Angiogenesis. Studying endothelial cell behavior in a three-dimensional culture system, consisting of extra cellular matrix proteins, allows for *in vitro* conditions that more closely mimic the *in vivo* environment permissive for cell differentiation into capillary-like structures. This assay system is called *in vitro* angiogenesis (40). To examine whether Figf/Vegf-D induces *in vitro* morphological changes resembling of capillary like-structure formation, endothelial cells were plated on a three-dimensional matrix of Matrigel (41) and then stimulated with increasing concentrations of Figf/Vegf-D. Endothelial cells grown under these conditions in the presence of 1% BSA exhibited a small round shape and did not spread. Treatment with Figf/Vegf-D for 24 h resulted in dramatic dose-dependent morphological changes. The cells became elongated, forming thin cords of intercon-

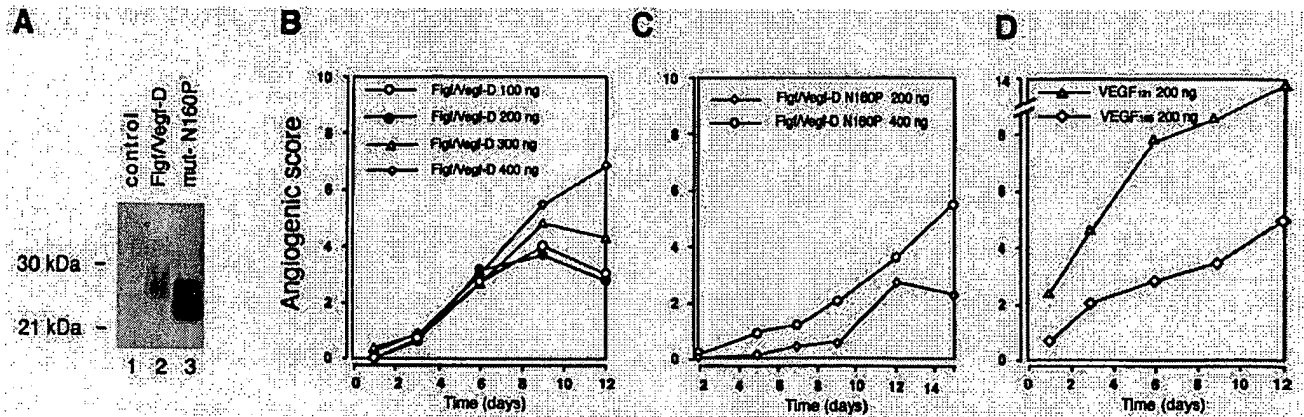


FIG. 2. Figt/Vegf-D sustains dose-dependent angiogenesis *in vivo*. (A) Supernatant of *S. cerevisiae* yeast strains expressing Figt/Vegf-D and Figt/Vegf-D mutant as indicated. (B) The angiogenic activity of various concentrations of Figt/Vegf-D were tested as slow-release preparations in the rabbit cornea assay. (C) Angiogenic activity of 200 and 400 ng/pellet of Figt/Vegf-D N160P. (D) Angiogenic activity of 200 ng/pellet of VEGF-A₁₂₁ and VEGF-A₁₆₅ is shown for comparison. Angiogenic score data are the mean values obtained from the responses scored in all animals in this study. Variations were below 10% of the mean values. Angiogenic scores are calculated as described in Fig. 1 and in *Materials and Methods*.

necting cells (Fig. 3). The effect was investigated in a range of concentrations between 5 and 200 ng/ml and was maximal at 100 ng/ml. Similar effects also were observed with 20 ng/ml VEGF-A. These data demonstrate that Figt/Vegf-D, like VEGF-A, is able to mediate dramatic cell reorganization, which would be necessary *in vivo* for the sprouting of endothelial cells and tube formation. No morphological alterations could be observed in KS-IMM cells, either by treating the cells with VEGF-A or with Figt/Vegf-D (not shown).

Figt/Vegf-D Induction of VEGFR-2 and VEGFR-3 Tyrosine Phosphorylation. It has been reported recently that VEGF-D and VEGF-C are the ligands of the endothelial tyrosine kinase receptors VEGFR-2 and VEGFR-3 (14, 23). To examine the

cellular response of endothelial cells to Figt/Vegf-D *in vitro*, we first tested whether Figt/Vegf-D could stimulate signal transduction from VEGFR-2 and VEGFR-3 in HUVECs and KS-IMM cells because these cells express both receptors. Tyrosine phosphorylation of these receptors was assayed in serum-starved cells treated with Figt/Vegf-D. VEGFR-2 and VEGFR-3 were immunoprecipitated with specific antibodies and analyzed by Western blotting with antiphosphotyrosine-specific antibodies. Figt/Vegf-D stimulated tyrosine phosphorylation of the 210-kDa VEGFR-2 and both the 125- and 195-kDa processed and unprocessed forms of VEGFR-3 in both HUVECs and KS-IMM cells (Fig. 4). Thus, Figt/Vegf-D, like VEGF-C, binds and activates these receptors on endothelial cells.

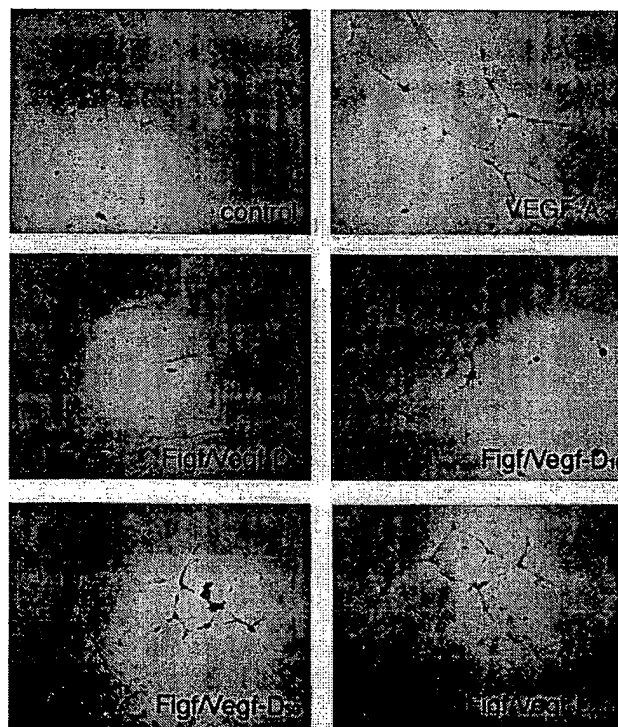


FIG. 3. Figt/Vegf-D-induced endothelial cell morphological changes. VEGF-A or Figt/Vegf-D were added to HUVECs cultured in three-dimensional Matrigel in low serum conditions. Photographs were taken 24 h after Figt/Vegf-D treatment. Protein concentrations (ng/ml) used are indicated.

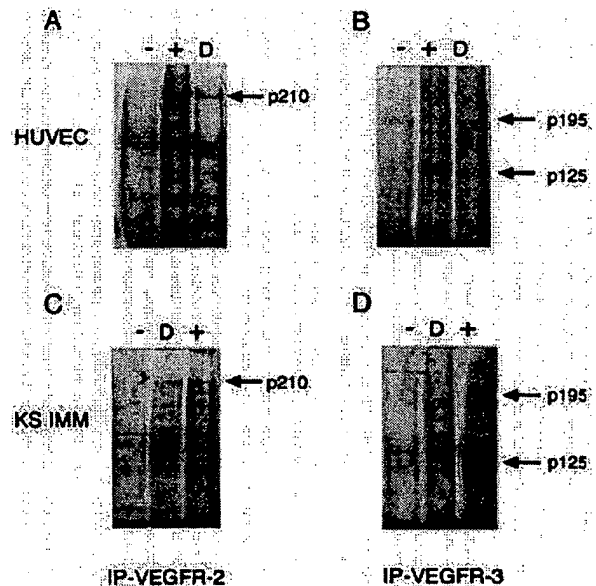


FIG. 4. Figt/Vegf-D-induced tyrosine phosphorylation of VEGFR-2 and VEGFR-3. HUVECs and KS-IMM cells were incubated with Figt/Vegf-D. After stimulation receptors were immunoprecipitated with antireceptor antibodies and analyzed by Western blotting with an antiphosphotyrosine mAb. (A and B) Phosphorylation of VEGFR-2 and VEGFR-3 in HUVECs. (C and D) Phosphorylation of VEGFR-2 and VEGFR-3 in KS-IMM cells. Positive control (+) and Figt/Vegf-D stimulation (D) is indicated. Arrows denote the position of the phosphorylated 210-kDa VEGFR-2 protein and the positions of the phosphorylated, proteolytically processed 125-kDa and unprocessed 195-kDa forms of VEGFR-3.

Fgf/Vegf-D Induction of Growth and Chemotaxis in HUVECs and KS-IMM Cells. To investigate further the proliferative effect of Fgf/VEGF-D on endothelial cells, we incubated both the cultured HUVECs and KS-IMM cells in the presence of increasing concentrations of Fgf/Vegf-D. Proliferation of both cell types was stimulated in a dose-dependent manner (Fig. 5 *A* and *B*). The effect was investigated in a range of concentrations between 5 and 100 ng/ml and was maximal at 50 ng/ml for both cell types. Interestingly, when suboptimal concentrations of VEGF-A₁₆₅ and Fgf/Vegf-D were coadded to HUVECs the resulting proliferation was higher than the treatment of each alone (not shown).

The chemotactic effect of Fgf/Vegf-D on HUVECs and KS-IMM cells was analyzed in a modified Boyden chamber assay. The migration of the cells through collagen-coated micropore filters toward chemoattractants was scored in the absence of serum. Fgf/Vegf-D stimulated the migration of KS-IMM cells in a dose-dependent manner. In HUVECs, under identical conditions Fgf/Vegf-D induced little or no migration (Fig. 5 *C* and *D*).

DISCUSSION

The results reported in this work show that Fgf/Vegf-D is a potent angiogenic factor. In rabbit corneas Fgf/Vegf-D, ex-

pressed either in CHO or yeast cells, can efficiently induce angiogenesis. The dose dependency and the early response suggest a direct effect of Fgf/Vegf-D on endothelial cell recruitment *in vivo*. This observation has been confirmed by *in vitro* experiments that show direct Fgf/Vegf-D activities on endothelial cells. In addition to Fgf/Vegf-D, other factors, including VEGF-A, basic fibroblast growth factor, placental growth factor, VEGF-C, and VEGF-E induce angiogenesis, suggesting a redundancy or a coordination among factors performing the same function (2, 10, 42, 43). The generation of VEGF-A knockout mice demonstrated that this factor is essential for angiogenesis during development (6, 7). Thus, simple redundancy of all members of the family is unlikely. We favor the hypothesis that the complex process of angiogenesis normally requires the cooperation of multiple factors and the experimental overexpression of some key members is able to trigger the process both directly and indirectly, inducing the expression of other factors. Each of these factors shows a peculiar pattern of expression, suggesting that a complex balance of factors in different developing organs may be relevant. Moreover, the biological function of VEGFs may not be limited to angiogenesis. For instance, fibroblast growth factors not only induce angiogenesis, but are also regulators of embryonic development, influencing the formation of several structures including body axis, limbs, heart, and lung differentiation (44–47). Similarly, the expression of Fgf/Vegf-D in tissues like the pituitary, the developing teeth, lung mesenchyme, and limb buds (26) suggests that Fgf/Vegf-D, in addition to playing a role in angiogenesis, could be involved in specific inductive signaling in these developing organs.

Fgf/Vegf-D and VEGF-C share striking similarities in their primary sequence and posttranslational modifications, and most importantly, both factors are recognized by VEGFR-2 and VEGFR-3 present on vascular and lymphatic vessels (14, 23). By using porcine aortic endothelial cells selectively overexpressing VEGFR-2 or VEGFR-3, it was shown recently that VEGF-C could promote migration and proliferation independently of signaling through either receptor (21). In this study for the analysis of Fgf/Vegf-D activity *in vitro* we used HUVECs and KS-IMM cells, which express both receptors. By immunoprecipitation experiments we showed that Fgf/Vegf-D activates both VEGFR-2 and VEGFR-3 tyrosine phosphorylation on both HUVECs and KS-IMM cells. The activation of these receptors by Fgf/Vegf-D stimulates a biological response that involves morphological, mitogenic, and motogenic responses. Thus, Fgf/Vegf-D shows a direct activity on endothelial cells *in vitro*, confirming the *in vivo* data. Although both HUVECs and KS-IMM cells show a similar mitogenic response to Fgf/Vegf-D they differ in motogenic responses because KS-IMM cells are more responsive than HUVECs to motogenic activation by Fgf/Vegf-D. This discrepancy could be simply because of differences in the receptor levels or in intracellular signaling molecules, but it also could be the result of the presence of possible coreceptors that may modify the receptor affinity and modulate the response to Fgf/Vegf-D. In line with this possibility, it recently has been shown in human endothelial cells that neuropilin-1 (48) and $\alpha\beta 3$ modulate the activity of VEGFR-2. Neuropilin-1 is a coreceptor for the VEGF-A₁₆₅ isoform and the $\alpha\beta 3$ integrin associates with VEGFR-1 upon VEGF-A stimulation and regulates the level of tyrosine phosphorylation of the receptor (49).

Fgf/Vegf-D differs from all other members of the VEGF family because it is the only angiogenic factor regulated by the nuclear oncogene *c-fos* (15). This unique regulation of Fgf/Vegf-D may be relevant both during development and in tumor progression because *c-fos* is involved not only in transformation but also in the regulation of cell growth and differentiation of various tissues (50, 51). Tumors that develop in *c-fos*-deficient mice appear devoid of vascularization although, in

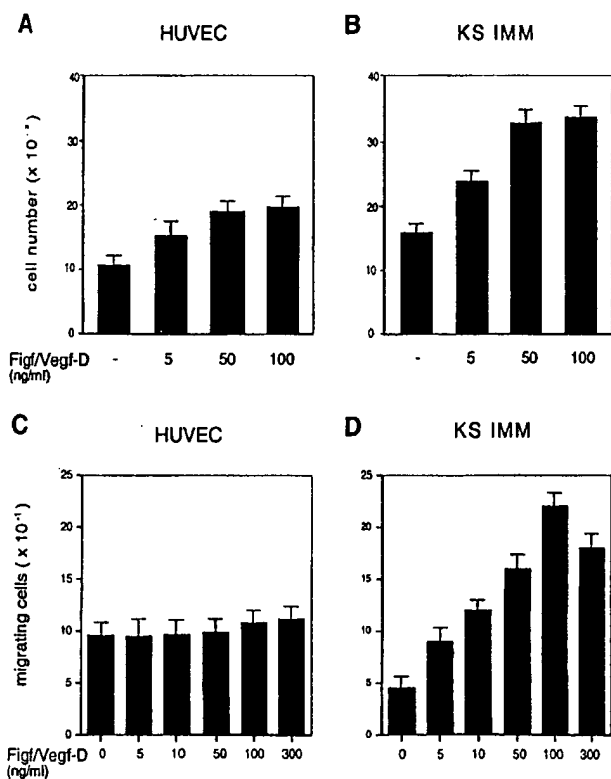


FIG. 5. Fgf/Vegf-D-induced cell proliferation and chemotactic activity. (*A* and *B*) Proliferative effects of Fgf/Vegf-D were assayed on HUVECs and KS-IMM cells as indicated. Experiments were performed in medium containing 1% FCS. After 72 h cells were enumerated by using a Coulter counter and values represent the mean (\pm SEM) of triplicate samples. (*C* and *D*) Cells were seeded in the upper wells of a 48-well micro chemotaxis Boyden chamber and incubated for 7 h at 37°C in medium containing 1% FCS. The lower wells contained the indicated concentrations of Fgf/VEGF-D. Cells migrating through a polycarbonate membrane with a pore size of 5 μ m were quantified by staining the cells with Giemsa solution and counting was performed on a light microscope of five high-power fields ($\times 100$). The results are expressed as the mean \pm 1 SD of three independent experiments performed in triplicate.

these papillomas, VEGF-A expression is reduced but not absent (52). The role of Figf/Vegf-D in tumor progression is at the moment unclear, although the evidence that Figf/Vegf-D shows angiogenic activity on endothelial cells, as well as mitogenic and motogenic activity on tumor-derived KS-IMM cells, strongly suggest that Figf/Vegf-D can be a *c-fos* effector for tumor malignancy.

We thank Nicholas Valiante for the critical reading of the manuscript, Rino Rappuoli for hospitality in IRIS laboratories, Cesira Galeotti for yeast strains and helpful discussions, and Astrid Parenti and Beatrice Grandi for technical assistance. This work was supported by funds from Italian Association for Cancer Research, Chiron Vaccines and CEE N0 BMH4-CT98-3380 (S.O.); the Italian Association for Cancer Research, Istituto Superiore di Sanità (AIDS Project; Program on Tumor Therapy), Centro Nazionale delle Ricerche (Progetto Finalizzato Biotecnologie), and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (60% and Programmi di Rilevante Interesse Nazionale) (F.B.), and the Italian Association for Cancer Research, Italian Ministry for University and for Scientific and Technological Research (Prot. 9706217225_06) and CEE PL 950669 (M.Z.). S.M. is supported by a grant from Centro Nazionale delle Ricerche (Progetto Biotecnologie).

- Ferrara, N. & Davis-Smyth, T. (1997) *Endocr. Rev.* **18**, 4-25.
- Nicosia, R. F. (1998) *Am. J. Pathol.* **153**, 11-16.
- Bussolino, F., Mantovani, A. & Persico, G. (1997) *Trends Biochem. Sci.* **22**, 251-256.
- Risau, W. (1997) *Nature (London)* **386**, 671-674.
- Folkman, J. (1997) *EXS* **79**, 1-8.
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kiechens, L., Gertsensein, M., Fairrig, M., Vandenhoeh, A., Harpal, H., Ebherardt, C., et al. (1996) *Nature (London)* **380**, 435-439.
- Ferrara, N., Carver-More, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. & Moore, M. W. (1996) *Nature (London)* **380**, 439-442.
- Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., et al. (1986) *Nature (London)* **320**, 695-699.
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. & Connolly, D. T. (1989) *Science* **246**, 1309-1312.
- Leung, D. W., Cachianes, G., Kuang, W., Goeddel, D. V. & Ferrara, N. (1989) *Science* **246**, 1306-1309.
- Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. & Persico, M. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9267-9271.
- Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K. & Erikson, U. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 2576-2581.
- Grimmond, S., Lagerkrantz, J., Drinkwater, C., Silins, G., Townson, S., Pollock, P., Gotley, D., Carson, E., Rakar, S., Norden-skjold, M., et al. (1996) *Genome Res.* **6**, 124-131.
- Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lathinen, I., Kuk, E., Saksela, O., Kalkkinen, N. & Alitalo, K. (1996) *EMBO J.* **15**, 290-298.
- Orlandini, M., Marconcini, L., Ferruzzi, R. & Oliviero, S. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 11675-11680.
- Rocchigiani, M., Lestingi, M., Luddi, A., Orlandini, M., Franco, B., Rossi, E., Ballabio, A., Zuffardi, O. & Oliviero, S. (1998) *Genomics* **47**, 207-216.
- Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y. & Shibuya, M. (1998) *J. Biol. Chem.* **273**, 31273-31282.
- Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., Lanz, C., Bettner, M., Rziha, H. J. & Dehio, C. (1999) *EMBO J.* **18**, 363-374.
- Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M. G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.-J., Chou, J. Y. & Persico, G. G. (1993) *Oncogene* **8**, 925-931.
- Cao, Y., Chen, H., Zhou, L., Chiang, M. K., Anand-Apte, B., Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G. & Tsang, M. L. (1996) *J. Biol. Chem.* **271**, 3154-3162.
- Cao, Y., Linden, P., Farnebo, J., Cao, R., Eriksson, A., Kumar, V., Qi, J.-H., Claesson-Welsh, L. & Alitalo, K. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14389-14394.
- Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K. & Alitalo, K. (1997) *Science* **276**, 1423-1425.
- Achen, M. G., Jeltsch, M., Kuk, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K. & Stacker, S. A. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 548-553.
- Yamada, Y., Nezu, J., Shimane, M. & Hirata, Y. (1997) *Genomics* **42**, 483-488.
- Jenkins, N. A., Woollatt, E., Crawford, J., Gilbert, D. J., Baldwin, M. E., Sutherland, G. R., Copeland, N. G. & Achen, M. G. (1997) *Chromosome Res.* **5**, 502-505.
- Avantaggiato, V., Acampora, D., Orlandini, M., Oliviero, S. & Simeone, A. (1998) *Mech. Dev.* **73**, 221-224.
- Kuk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V. & Alitalo, K. (1996) *Development (Cambridge, U.K.)* **122**, 3829-3837.
- Enholm, B., Paavonen, K., Ristimäki, A., Kumar, V., Gunji, Y., Klefstrom, J., Kivinen, L., Laiho, M., Olofsson, B., Jukov, V., et al. (1997) *Oncogene* **14**, 2475-2483.
- Ristimäki, A., Narko, K., Enholm, B., Joukov, V. & Alitalo, K. (1998) *J. Biol. Chem.* **273**, 8413-8418.
- Baldari, C., Murray, J. A., Ghiara, P., Cesareni, G. & Galeotti, C. (1987) *EMBO J.* **6**, 229-234.
- Ziche, M., Alessandri, G. & Gullino, P. M. (1989) *Lab. Invest.* **61**, 629-634.
- Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H. T., Donnini, S., Granger, H. J. & Bicknell, R. (1997) *J. Clin. Invest.* **99**, 2625-2634.
- Bussolino, F., Wang, J. M., Defilippi, P., Turrini, F., Sanavio, F., Edgell, C. J. S., Aglietta, M., Arese, P. & Mantovani, A. (1989) *Nature (London)* **337**, 471-473.
- Albini, A., Paglieri, I., Orenco, G., Carlone, S., Aluigi, M. G., DeMarchi, R., Matteucci, C., Mantovani, A., Carozzi, F., Donini, S. & Benelli, R. (1997) *AIDS* **11**, 713-721.
- Kleinman, H. K., McGarvey, M. L., Hassel, J. R., Star, V. L., Cannon, F. B., Laurie, G. W. & Martin, G. R. (1986) *Biochemistry* **25**, 312-318.
- Keung, K., Silfer, E. & Eppenberger, V. (1989) *Anal. Biochem.* **182**, 16-19.
- Dejana, E., Languino, L. R., Polentarutti, N., Balconi, C., Ryckewaert, J. J., Larrieu, M. J., Mantovani, A. & Marguerie, G. (1985) *J. Clin. Invest.* **75**, 11-18.
- Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N. & Alitalo, K. (1997) *EMBO J.* **16**, 3898-3911.
- Morbidelli, L., Birkenhaeger, R., Roeckl, W., Granger, H. J., Kaerst, U., Weich, H. & Ziche, M. (1997) *Angiogenesis* **1**, 117-130.
- Montesano, R., Orci, L. & Vassalli, P. (1983) *J. Cell. Biol.* **97**, 1648-1652.
- Grant, D. S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G. R. & Kleinman, H. K. (1989) *Cell* **58**, 933-943.
- Basilico, C. & Moscatelli, D. (1992) *Adv. Cancer. Res.* **59**, 115-165.
- Ziche, M., Maglione, D., Ribatti, D., Morbidelli, L., Lago, C. T., Battisti, M., Paoletti, I., Barra, A., Tucci, M., Parise, G., et al. (1997) *Lab. Invest.* **76**, 517-531.
- Szebenyi, G. & Fallon, J. F. (1999) *Int. Rev. Cytol.* **185**, 45-106.
- Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B. & Mignatti, P. (1998) *J. Cell. Biol.* **141**, 1659-1673.
- Mima, T., Ueno, H., Fischman, D. A., Williams, L. T. & Mikawa, T. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 467-471.
- Peters, K., Werner, S., Liao, X., Wert, S., Whitsett, J. & Williams, L. (1994) *EMBO J.* **13**, 3296-3301.
- Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. & Klagsbrun, M. (1998) *Cell* **92**, 735-745.
- Soldi, R., Mitola, S., Strasys, S., Defilippi, P., Tarone, G. & Bussolino, F. (1999) *EMBO J.* **18**, 882-892.
- Johnson, R. S., Spiegelman, B. M. & Papaioannou, V. E. (1992) *Cell* **71**, 577-586.
- Wang, Z.-Q., Ovitt, C., Gregoriadis, A. E., Möhle-Steinlein, U., Rüther, U. & Wagner, E. F. (1992) *Nature (London)* **360**, 742-745.
- Saez, E., Rutemberg, S. E., Mueller, E., Oppenheim, H., Smolak, J., Yuspa, S. H. & Spiegelman, B. M. (1995) *Cell* **82**, 721-732.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ashkenazi et al.)	Group Art Unit 1724
Appl. No.	:	10/066,273)	
Filed	:	February 1, 2002)	
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME)	
Examiner	:	OLGA N. CHERNYSHEV)	

DECLARATION OF MARY GERRITSEN, Ph.D. UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Mary Gerritsen, Ph.D. declare and state that:

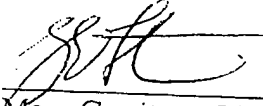
1. I am a co-inventor of the invention described in U.S. Patent Application Serial No. 10/066,273 entitled SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME.
2. My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).
3. From 1997-2001 I worked for Genentech as a Senior Scientist in the Department of Cardiovascular Research. During this time I directed and analyzed various bioassays and numerous molecular biology techniques including microarray analyses. In 2002, I accepted a position as Senior Director of Vascular Biology and Functional Genomics at Millennium Pharmaceuticals. Currently I am employed as the Senior Director of Molecular Pharmacology at Exelixis. These positions have provided me with extensive experience in vascular research, including angiogenesis and cancer development.

4. I am familiar with the specification and claims of U.S. Patent Application Serial No. 10/066,273, both the outstanding Office Action mailed September 17, 2004 and the first Office Action mailed April 28, 2004, and the issues raised therein.
5. The specification of U.S. Patent Application Serial No. 10/066,273 describes Assay 93 in Example 60. Assay 93 was performed to determine whether particular compounds are capable of inducing c-fos expression in pericyte cells. As stated in Example 60, the novel polypeptide PRO444 tested positive in this assay. The results and significance of Assay 93 are described in more detail herein in an effort to provide the U.S. Patent and Trademark Office (USPTO) with more information regarding the significance of c-fos induction in pericyte cells.
6. Assay 93 is an assay designed to determine whether particular compounds are capable of stimulating retinal pericytes through the c-fos pathway. Retinal pericytes are unique cells that play an important role in regulating angiogenesis. More specifically, pericytes help regulate capillary permeability and stabilize newly formed blood vessels. C-fos is a transcription factor involved in the regulation of cellular growth, including cancer and angiogenesis. Growth factors capable of stimulating pericytes signal through the c-fos pathway.
7. In light of their significant relationship with angiogenesis and cancer, it is useful to identify compounds capable of stimulating pericytes through the c-fos pathway in order to treat, promote and diagnose these conditions. Furthermore, one with skill in the art would reasonably conclude that the presence or overexpression of a compound capable of inducing c-fos expression in pericytes (e.g., PRO444) in a subject would more likely indicate the onset of cancer and/or angiogenesis as opposed to a subject who lacked this polypeptide. Likewise, a skilled artisan would also reasonably conclude that neutralizing compounds capable of stimulating c-fos expression in pericytes (e.g., PRO444) could be useful in preventing the onset and/or progression of cancer and/or angiogenesis.
8. In the outstanding Office Action, the Examiner alleged that with respect to the positive results observed when PRO444 was tested in Assay 93, "one skilled in the art would not attribute the induction of c-fos expression in pericytes by [PRO444] as a physiological reaction specifically induced by [PRO444]." (Office Action, page 3). On the contrary, Assay 93 included both positive and negative test controls: DME + 5% serum +/- PDGF and buffer respectively. The use of these controls ensured that the resulting data were attributed to the specifically tested compounds (e.g., PRO444), as opposed to some other factor or stimulus. Accordingly, a skilled artisan would readily have attributed the detected c-fos induction specifically to the PRO444 polypeptide.
9. In the first Office Action mailed April 28, 2004, the Examiner cited three journal articles: Janknecht et al., *Carcinogenesis*, vol. 16 no. 3, pp. 443-450 (1995), Herrera et al., *Progress in Neurobiology*, vol. 50, pp. 83-107 (1996), and Kovács, *Neurochem Int.* vol. 33, pp. 287-297 (1998) to support the assertion that c-fos induction is a "non-specific first line of cellular response" and that PRO444 accordingly lacks sufficient utility. It is important to note that none of these three articles discuss whether c-fos induction in

pericyte cells is a general response. For example Kovács is directed to c-fos induction in neuronal cells, and Herrera et al. is directed to c-fos expression in brain cells. Accordingly the teachings of these articles regarding c-fos induction are not necessarily applicable to pericytes, the specific cell type tested in Assay 93.

10. In Assay 93, 646 samples representing 382 different compounds were tested for their ability to induce c-fos expression in pericytes. The tested compounds included many known cytokines (e.g., Interleukin-1, tumor necrosis factor, interferon), growth factors (e.g., vascular endothelial growth factor, fibroblast growth factor, epidermal growth factor), chemokines, autocoids (e.g. endothelin), hormones (e.g. glucagons, luteinizing hormone) and polypeptides of unknown function. Of the 646 different samples that were assayed, only 48 tested positive for inducing c-fos expression in pericyte cells. Several of the 48 samples testing positive represented different lots of the same compound. As very few of the tested compounds were able to induce c-fos expression, it can be reasonably concluded from these results that the stimulation of c-fos in pericytes is not a generalized response.
11. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Jan 15/2005


 Mary Gerritsen, Ph.D.

CURRICULUM VITAE
Mary E. Gerritsen, Ph.D.

Residence

Address: 541 Parrott Drive
San Mateo Ca 94402
Home phone 650 348 6492
meg570@comcast.net

Education:

- | | |
|------|--|
| 1975 | Bachelor of Science
University of Calgary
Calgary, Alberta Canada
Summa Cum Laude (Zoology) |
| 1978 | Doctor of Philosophy, Endocrinology/Pharmacology.
University of Calgary, Faculty of Medicine.
Calgary, Alberta Canada. |

Postdoctoral Training:

- | | |
|-----------|--|
| 1978-1980 | Research Pharmacologist, Department of Pharmacology,
University of California, San Diego. |
|-----------|--|

Academic Appointments:

Department of Physiology, New York Medical College

- | | |
|-----------|---|
| 1980-1986 | Assistant Professor of Physiology |
| 1986-1989 | Associate Professor of Physiology. |
| 1986 | Associate Professor of Physiology with Tenure |
| 1990-1996 | Adjunct Associate Professor |

Pharmaceutical Industry Appointments:

Miles Pharmaceuticals (Renamed Bayer Corporation, April 1995)

- | | |
|-----------|--|
| 1990-1995 | <u>Senior Staff Scientist</u> ; Institute for Inflammation and Autoimmunity <ul style="list-style-type: none">▪ Led screening efforts for small molecule inhibitors of leukocyte adhesion inhibitors |
|-----------|--|

- Identified flavonoids as potent inhibitors of cytokine induced gene expression
- Identified first synthetic inhibitors of IKB kinase, BAY 11-7082 and 11-7085.

1990-1992

Group Leader, Leukocyte Immigration.

- Coordinated screening efforts on MMP inhibitors for rheumatoid arthritis. Clinical candidate identified and developed for cancer metastasis
- Championed screen development for p38 MAP kinase. Program initiated and potent compounds identified
- Supervised group of four laboratories, (5 Ph.D.s and 9 Ras)
- Initiated reporter gene and transcription factor screens for inflammation targets
- Identified potent ICAM-1 blocking antibody
- Coordinated development of lipid mediator program. Developed screens for cyclooxygenase I and II inhibitors
- Worked with multidisciplinary teams including chemistry, pharmacokinetics, metabolism, formulation and pre-clinical development on inflammation projects-MMP inhibitors, p38 MAP kinase inhibitors, cytokine inhibitors, leukocyte adhesion inhibitors, cyclooxygenase inhibitors
- Evaluated in-licensing opportunities for small molecules in inflammation, osteoarthritis and osteoporosis

1992-1996

Arbeitskreis Moderator (Similar to Associate Director), Inflammation Research

- Coordination of all research discovery programs in Inflammation involving both internal and external research groups
- Supervised Six Arthritis Laboratories (8 Ph.d.s, 16-20 RAs)
- Presentation of research progress at quarterly in house meetings and at annual Bayer-wide meeting (held in Germany). Evaluation of other programs
- Evaluation of various in-licensing opportunities
- Wrote NDA for ketoprofen analog in-licensed by Bayer
- Recruited and built interdisciplinary group in Rheumatoid Arthritis and Chronic Inflammatory Disease
- Researched and wrote strategic plan and competitive assessment

1993-1997

Principal Staff Scientist, Inflammation Research

- Continued to support screening programs for NF- κ B inhibitors
- Developed external collaborations with Vascular Research Division at Brigham and Womens Hospital-laboratories of Drs. Francis W. Luscinskas, Michael Gimbrone and Tucker Collins.

- Identified and mapped interaction of the coactivators CBP/p300 with NF- κ B
- Led target validation team for Ceramide/Sphingomyelinase in Osteoarthritis

Genentech Inc.

1997- 2001

Senior Scientist, Department of Cardiovascular Research

- Initiated the development of an angiogenesis target discovery program using Curagen™ technology and Affymetrix oligonucleotide arrays
- Coordinated the screening efforts of multiple groups working on various aspects of vascular biology for SPDI (Secreted Protein Discovery Initiative)
- Evaluated various in-licensing opportunities for Cardiovascular Research, Oncology and Immunology
- Coordinated external collaboration with Dr. Alexander Clowes, University of Washington on EGF receptors and restenosis
- Served on preclinical development committee for VEGF-Therapeutic angiogenesis
- Served on Clinical Development committee for CD18 monoclonal antibody
- Identified critical roles for PECAM and VE-Cadherin in endothelial differentiation into tube-like structures
- Identified PPAR γ ligands as potent inhibitors of growth factor induced angiogenesis
- Identified critical role of hepatocyte growth factor in endothelial differentiation in vitro and angiogenesis in vivo
- Demonstrated that KDR (VEGF receptor) plays essential role in endothelial cell differentiation into tube like structures
- Identified over 100 novel targets for either promotion or inhibition of angiogenesis
- Identified stanniocalcin as a novel angiogenic modulator
- Used affymetrix oligonucleotide arrays to identify critical angiogenesis progression factors in renal cell carcinoma
- Identified critical roles for matrix metalloproteinases and c-src in capillary lumen formation
- Identified a novel peptide fragment that may be a key player in inflammation and angiogenesis.

2000

Acting Director, Department of Cardiovascular Research

- Coordinated all department efforts in research discovery and preclinical development
- Continued to coordinate angiogenesis target discovery initiative. Discovered several novel molecules with key roles in endothelial differentiation in vitro, angiogenesis in vivo, and regulators of vascular permeability
- Evaluated various in-licensing opportunities
- Worked on Therapeutic Area Focus committee to define new directions for Cardiovascular Research
- Initiated a Cardiovascular Research Seminar series to bring in outside speakers on a biweekly basis. Coordinated collaborations that resulted from this initiative
- Head, Cardiovascular Recruitment committee. Organized successful search for Senior Scientist level positions.

2000-2001 Associate Director, Department of Cardiovascular Research, Genentech

- Researched and developed strategic plan for Department with Director
- Coordinated projects with internal and external research groups
- Provided scientific support to clinical and marketing groups
- Continued projects initiated above

2002-2003 Senior Director, Departments of Vascular Biology and Functional Genomics

Millennium Pharmaceuticals, Inc. South San Francisco CA

- Served as project leader on three small molecular discovery programs at different stages-hit to lead, late development, high throughput screening
- Supervised vascular biology staff (four senior scientists, two post-doctoral fellows, 6 research associates, associate scientists and research investigators)
- Supervised histology core facility (one scientist and one research associate)
- Supervised functional genomics group at South San Francisco (two scientists at MSF), helped to coordinate activities with Millennium Cambridge facility.
- Developed strategic plan for vascular biology effort at Millennium
- Initiated large scale genomic screening program for targets in atherosclerosis, aortic aneurysm, and diabetic vascular disease as well as lung and renal fibrosis

- Initiated collaborations with over 10 academic laboratories in animal model development, human and primate disease models
- Served on pharmacology working group committee to oversee small molecule evaluation in vivo models
- Presented status updates to senior management at Scientific Review Committee meetings on a quarterly basis
- Coordinated MSF efforts in Bayer collaboration-Qualified Target initiative
- Developed and characterized animal models for drug screening programs.
- Worked on a biomarker initiative for each of our screening programs combining genomic analysis and mechanism of action studies.

2004-present Senior Director, Molecular Pharmacology. Exelixis South San Francisco, CA

- Supervise 4 associate directors (total group of 30)
- Direct all cell-based screens and pharmacodynamic studies to support projects in oncology, metabolic disease and inflammation
- Evaluate lead validation and lead optimization programs
- Establish outsourced pharmacology studies to support internal programs
- Identify new molecular targets for New Lead Discovery high through put screens

Projects and Research Areas of Expertise:

Eicosanoid Metabolism and Physiology

Adhesion Molecules

Mechanism of Drug Action

Matrix Metalloproteinases

Cell Based and Molecular Screening

Transcription Factors, Promoter Analysis

Endothelial Cell Biology, General Cell Biology

Vascular Biology

Microcirculation

Angiogenesis

Gene Expression Profiling using Differential Display, Affymetrix Arrays

Rosetta Resolver Software for microarray analysis

Functional Genomics

Rheumatoid Arthritis, Chronic Inflammatory Diseases

Atherosclerosis

Coronary, Peripheral and Cerebral Cardiovascular Disease

Macular Degeneration
Diabetic retinopathy
Models of fibrosis (lung, renal, liver)
Cell based screening for oncology, metabolism and inflammation

Awards and Honors:

Province of Alberta Graduate Scholar 1976
Medical Research Council Studentship 1976-1978
Isaac Walton Killam Scholar and Merit Award 1977,1978
Medical Research Council Fellow 1978-1980
Alexander and Alexandrine Sinsheimer Scholar 1981, 1982
Pharmacia Young Investigator Award, Microcirculatory Society 1983
Mary Weideman Award, Microcirculatory Society 1984
NIH Research Career Development Award 1987-1992
Miles Science Award 1992
Kurt Weiderhelm Award, Microcirculatory Society 1998
Award named after me (Gerritsen Award), awarded annually by the Microcirculatory Society

Major Committee Assignments

National and Regional:

1985-	Ad hoc Grant Reviewer/Site Visitor: Experimental Cardiovascular Sciences Study Section (NIH)
1986-	Ad hoc reviewer VA Intramural Research Program
1987-	Ad hoc reviewer Medical Research Council of Canada, Canadian Heart Foundation, New York State Heart Association
1992-2001	Member, NIH Pathology A Study Section
1989-1992	Council, Microcirculatory Society
1991	Nominations Committee, Microcirculatory Society
1992	Chairperson, Publications Committee, Microcirculatory Society
1992	Liason Committee, American Physiology Society
1993	Steering Committee, North American Vascular Biology Organization

	Convenor, Blood Vessel Club, Anaheim CA
1994	Councillor, North American Vascular Biology Organization
1995	International Advisory Committee, 2nd Asian Microcirculation Meeting,
1996-	Co-Chairman, Keystone Symposia on Oxidant Stress, From Molecules to Man, Santa Fe NM
1997,1998,2001	Program Committee, Vascular Biology '98 and Vascular Biology '99, ATVB meeting 2001
2000	Vascular Biology Study Section, American Heart Association President, North American Vascular Biology Organization Development Committee, North American Vascular Biology Organization International Advisory Committee , World Congress of Microcirculation
2001	Coorganizer, with Richard Hynes and Denisa Wagner of Keystone Conference on Angiogenesis and Chronic Disease
2001	NIH Program Project Site Visit Team, National Cancer Institute
2001	NIH Stroke Progress Review Group
2001	External Scientific Advisory Committee, Institute for Medicine and Engineering, University of Pennsylvania
2002	Organized Career Symposium "Women in Industry" at the Exp. Biol.
2003-present	Development Committee Chair, North American Vascular Biology Organization

New York Medical College:

1980-1989	Member, Graduate Faculty (elected)	
1980-1985	Student Life Committee	
1980-1989	Safety Committee	
1980-1990	Member, Search Committees for Chairmen, Departments of	Cardi
1987-1989	Tenure and Promotions Committee	

Bayer Corporation:

1992	Co-Chairman, Bayer International Adhesion Meeting, Cologne Germany
1993-	Diversity Committee
1995-1996	Safety Committee

Editorial Boards:

1993-1998	Founding Editor and Editor in Chief, MICROCIRCULATION, the official journal of the Microcirculatory Society
1998-	Consulting Editor, MICROCIRCULATION
1999-	Editorial Board, ENDOTHELIUM, JOURNAL OF ENDOTHELIAL RESEARCH
1992-2000	Editorial Board, AMERICAN JOURNAL OF PHYSIOLOGY (Heart and Circulation)
1989-1995	Associate Editor, MICROVASCULAR RESEARCH
1993-2000	Editor, North American Vascular Biology Organization (NAVBO) Newsletter; Co-editor, NAVBO WWW Home Page
1995-present	Editorial Board, JOURNAL OF CARDIOVASCULAR PATHOLOGY
1996-2000	Editorial Board, CIRCULATION RESEARCH

Memberships in Professional Societies:

American Association for the Advancement of Science
American Horticultural Society
American Physiological Society
American Society for Pharmacology and Experimental Therapeutics
American Society for Research on Vision and Ophthalmology
American Society for Investigational Pathology
The Microcirculatory Society, Inc .
North American Vascular Biology Organization (NAVBO)
American Society for Cell Biology
Society for Leukocyte Biology
Peninsula Orchid Society
American Orchid Society
Pleurothallid Alliance

AHA Council on Arteriosclerosis, Thrombosis and Vascular Biology
Fellow of the American Heart Association
AHA Council on Stroke

Summary of Teaching Experience:

A. Courses

University of Calgary:

Pharmacology

Lecturer, Endocrinology 1977, 1978

University of California, San Diego:

Physiology/Pharmacology

Teaching Assistant and Laboratory Instructor 1979, 1980
(Pharmacokinetics, Metabolism labs)

New York Medical College:

Human Physiology

Lecturer (endocrinology) 1980-1990

Molecular Endocrinology

Course Director and Lecturer 1980-1990

Methods in Endocrinology

Course Director 1986

Cells of the Vessel Wall

Course Director 1986, 1988

Biochemical Pharmacology

Lecturer 1980-present (receptor pharmacology, cell culture, eicosanoid
biochemistry, biologicals as drugs)

Review courses (Cardiovascular, Endocrinology) for Medical Boards at Bellvue
Hospital, NY and other NY Medical College affiliate hospitals.

Jefferson Medical College:

Graduate Course in Human Physiology

Lecturer 1985-1987 (vascular cell biology, eicosanoid biochemistry)

City College of New York

Human Physiology

Lecturer 1980-1989 (Endocrinology)

University of Virginia

Shaking the Academic Tree: Alternative Careers 1999

B. Research Supervision

Predoctoral research experiences (summers, elective periods): New York Medical College graduate and medical students
Doctoral Research Advisor/Supervisor: New York Medical College Physiology, Pharmacology, Cell Biology
Postdoctoral Supervisor: New York Medical College and Miles/Bayer Corporation
Postdoctoral Supervisor: Genentech
Summer Intern Advisor, Genentech

Sponsored Research Programs (Principal Investigator)

National Institutes of Health

1981-1984	NIH New Investigator Award "Cerebral Microvessels "
1985-1988	NIH HLBI RO1 Grant "Glucocorticoids and Microvessel Endothelium"
1985-1988	NIH EI RO1 Grant "Retinal Endothelial Cells"
1986-1989	Research Career Development Award (NIH, returned in 1990)
1990-1996	NIH HLBI RO1 grant "Glucocorticoids and Microvessel Endothelium"

Other Agencies

1981-1984	American Heart Association Grant-in-Aid "Isolation and Characterization of Endothelial Cells from Cardiac Muscle"
1984	American Diabetes Association Grant-in-Aid "Effects of High Glucose on Retinal Microvascular Endothelial Cells"
1984-1986	Westchester Heart Association Grant-in-Aid " Effects of High Glucose on Cardiac Muscle Microvessel Endothelial Cells"
1984-1985	New York State Health Research Council Grant-in-Aid "Eicosanoid Metabolism in Cardiac Muscle Microvessel Endothelium"
1986	Boehringer Ingelheim Grant-In-Aid "Isolation of a Leukocyte Regulatory Factor from Microvessel Endothelium"
1990	Miles Inc. Grant in Aid. Fellowship support for Robert Mannix
1989	Fight for Sight Fellowship (sponsor for Julio Rimarachin)
1989	New York Eye and Ear Fellowship (sponsor for Julio Rimarachin)

Consultantships:

1986-1989	INSITE VISION, Alameda California
1986-1989	Boehringer Ingelheim Pharmaceuticals, Ridgefield CT

2001	Department of Vascular Medicine, Stanford CA
2003-2004	Frazier HealthCare Ventures, Palo Alto, CA
2003	Xoma Corporation, Berkeley, CA
2004-	Macusight, Freemont, CA

Students and fellows supervised and their current positions:

1981-1985	Terry O Meyers, Ph.D. Associate Professor of Physiology, City University of New York
1985-1987	Robert Gundel, Ph.D. Vice President, Pre-clinical Research, Xoma Corporation
1984-1988	Anthony Capetandes, Ph.D. Scientist, Merck
1981-1983, 1987	Richard Rosenbaum, M.D. Fellow, Department of Cardiology, Jefferson Medical College, Philadelphia
1986-1989	Catherine Partridge, Ph.D. Associate Professor, Department of Biochemistry, Albany Medical College
1986-1989	Julio A. Rimarachin, M.D. Associate Professor, Cornell University Medical College
1985-1989	Robert Mannix, Ph.D. Research Cell Biologist, Children's Hospital, Harvard University
1988-1993	Tariq Moatter, Ph.D. Assistant Professor, Aga Khan University, Karachi Pakistan
1993-1999	Eric Schwartz, Ph.D. Post-doctoral fellow, Stanford University
1998	Jennifer Graham, Orthopedics resident, Brigham and Women's Hospital
1998-2000	Xiaohua Xin, Scientist, Eli Lilly
1999-present	Hainsworth Shin, Post-doctoral fellow, UCSD
2000-present	Max Tejada, Post-doctoral fellow, Genentech

Bibliography

Original Research Reports

1. **Gerritsen, M.E.** and Lederis K. Effects of urotensin I on intracellular levels of cAMP in the rat tail artery. *Eur. J. Pharmacol.* 60:211-219, 1979.
2. **Gerritsen, M.E.** and Lederis, K. Effects of urotensin I on the isolated rat tail artery. *Pharmacology.* 18:72-79, 1979.
3. **Gerritsen, M.E.**, Parks, T.P., Printz, M.P. Prostaglandin endoperoxide metabolism in bovine cerebral microvessels. *Biochim. Biophys. Acta* 619:196-206, 1980.
4. **Gerritsen, M.E.** and Printz, M.P. Prostaglandin E₂ synthesis in pigeon aorta: comparison of atherosclerosis-resistant (show racer) and atherosclerosis-prone (white carneau) pigeon breeds. *Artery* 8:56-62, 1980.
5. **Gerritsen, M.E.** and Printz, M.P. Sites of prostaglandin synthesis in the bovine heart and isolation of coronary microvessels. *Circ. Res.* 49:1159-1171, 1981.
6. **Gerritsen, M.E.** and Printz, M.P. PGD synthase in microvessels from the rat cerebral cortex. *Prostaglandins* 22:553-557, 1981.
7. **Gerritsen, M.E.**, Morgan, D.O.M., Parks, T.P., Printz, M.P. and Lederis, K. A proposed role for prostaglandins in the modulation of the relaxation response to urotensin I in isolated rat arteries. *Prostaglandins* 22:873-892, 1981.
8. **Gerritsen, M.E.** PGD₂ formation in the vasculature. Characteristics of rat tail vein PGH-PGD isomerase. *Prostaglandins* 25:105-120, 1983.
9. **Gerritsen, M.E.** and Cheli, C.D. Arachidonic acid and prostaglandin endoperoxide metabolism in isolated rabbit coronary microvessels and isolated cultivated coronary microvessel endothelial cells. *J. Clin. Invest.* 72:1658-1671, 1983.
10. Rodrigues, A.M. and **Gerritsen, M.E.** Release of 6-keto PGF_{1α} and PGE₂ from isolated rabbit cerebral microvessels: effects of 100% O₂ room air and 95% N₂ 5% CO₂. *Stroke* 15:717-722, 1984.
11. Levine, N., Tarlin, N. and **Gerritsen, M.E.** Effect of castration on prostaglandin mediated changes in membrane potential and prostaglandin synthesis in guinea pig seminal vesicles. *J. Reprod. Fertil.* 73:539-545, 1985.

12. Myers, T.O.M., Messina, E.J., Rodrigues, A.M. and **Gerritsen, M.E.** Altered prostaglandin synthesis in the cremaster muscle and aorta from streptozotocin-induced diabetic rats. *Am. J. Physiol.* 249:E374-379, 1985.
13. **Gerritsen, M.E.** and Burke, T. Insulin binding and effects of insulin on glucose metabolism and 2-deoxyglucose uptake in isolated rabbit coronary microvessel endothelial cells. *Proc. Soc. Exp. Biol Med.* 180:17-23, 1985.
14. Rosenbaum, R. and **Gerritsen, M.E.** Effects of dexamethasone on rabbit coronary microvessel endothelial (RCME) cell prostaglandin synthesis. *Am. J. Physiol.* 250:C970-977, 1986.
15. Allen, L.A. and **Gerritsen, M.E.** Regulation of hexose transport in cultured bovine retinal microvessel endothelium by insulin. *Exp. Eye Res.* 43:679-686, 1986.
16. **Gerritsen, M.E.**, Weinstein, B.I., Gordon, G.G. and Southren, A.L. Prostaglandin synthesis and release from cultured human trabecular meshwork cells and scleral fibroblasts. *Exp Eye Res.* 43:1089-1102, 1987.
17. **Gerritsen, M.E.**, Ngalene, D.M. and Rodrigues, A.M. Calcium ionophore (A23187) and arachidonic acid stimulated prostaglandin release from microvascular endothelial cells: effects of calcium antagonists and calmodulin inhibitors. *J. Pharm. Exp. Ther.* 240:837-846, 1987.
18. Belloni, F.L., Liang, B.C. and **Gerritsen, M.E.** Effects of alkylxanthines and calcium antagonists on adenosine uptake by cultured rabbit coronary microvessel endothelium *Pharmacol.* 35:1-15, 1985.
19. Churchill, L., Buasback, H., **Gerritsen, M.E.** and Ward, P.E. Metabolism of opoid peptides by cerebral microvasculature aminopeptidase. *Biochim. Biophys. Acta* 923:35-41, 1987.
20. **Gerritsen, M.E.** Eicosanoid production by the coronary microvascular endothelium. *Fed. Proc.* 46:47-53, 1987.
21. **Gerritsen, M.E.** Functional heterogeneity of vascular endothelium. *Biochemical Pharmacology.* 36:2701-2711, 1987.
22. Gundel, R.H., **Gerritsen, M.E.**, Gleich, G.J. and Wegner, C.D. Repeated antigen inhalation results in a prolonged airway eosinophilia and airway hyperresponsiveness in primates. *J. Appl. Physiol* 68:779-786, 1990.
23. **Gerritsen, M.E.**, Burke, T. and Allen, L. Glucose starvation is required for insulin stimulation of hexose uptake and metabolism in coronary microvascular endothelial cells. *Microvascular Research* 35:153-166, 1988.

24. Gundel, R.H., **Gerritsen, M.E.**, and Wegner, C.D. Antigen coated sepharose beads induce airway eosinophilia and airway hyperresponsiveness in cynomologous monkeys. *Am. J. Resp. Dis.* 140:629-633, 1989.
25. Medow, M.E., Intrieri, L., Moatter, T. and **Gerritsen, M.E.** Dexamethasone effects on membrane lipid composition of microvascular endothelial cells. *Am. J. Physiol.* 257:C512-519, 1989.
26. Partridge, C.A., **Gerritsen, M.E.**, Southren, A.L. and Weinstein, B.I. Dexamethasone induces specific proteins in human trabecular meshwork cells. *Invest. Ophthalmol. Vis. Sci.* 30:1843-1847, 1989.
27. **Gerritsen, M.E.**, Rimarachin, J., Perry, C.A. and Weinstein, B.I. Arachidonic acid metabolism by cultured bovine corneal endothelial cells. *Invest. Ophthalmol. Vis. Sci.* 30:698-705, 1989.
28. **Gerritsen, M.E.**, Perry, C.A. Moatter, T. Cragoe, E.J.jr and Medow, M.S. Role of Na^+/H^+ antiport in agonist specific prostaglandin release from microvessel endothelium. *Am. J. Physiol.* 256:C831-839, 1989.
29. Partridge, C.A. and **Gerritsen, M.E.** Dexamethasone increases the release of three 44 kD proteins immunologically related to plasminogen activator-inhibitor-1 from HUVE and RCME cells. *Thromb. Res.* 57:139-154, 1990.
30. Capetandes, A. and **Gerritsen, M.E.** Simplified methods for the selective and consistent culture of bovine retinal endothelial cells and pericytes. *Invest. Ophthalmol. Vis. Sci.* 31:1738-1744, 1990.
31. **Gerritsen, M.E.** and Perry, C.A. Regulation of eicosanoid synthesis in microvessel endothelium: glucocorticoids do not effect arachidonoyl CoA synthase activity. *Biochim. Biophys. Acta* 1045:174-179, 1990.
32. Masferrer, J.L., Rimarachin, J.A., **Gerritsen, M.E.**, Falck, J.R., Yadagiri, P., Dunn, M.W. and Lanaiido-Schwartzman, M.. 12 (R) hydroxyeicosatrienoic acid, a potent chemotactic and angiogenic factor produced by the cornea. *Exp. Eye Res.* 52:417-424, 1991.
33. **Gerritsen, M.E.**, Schwarz, S.M., and Medow, M.S. Glucocorticoid-mediated alterations in fluidity of rabbit cardiac muscle microvessel endothelial cell membranes: Influences on eicosanoid release. *Biochim. Biophys. Acta* 1065:63-68, 1991.
34. Koller A., Sayedi, N, **Gerritsen, M.E.** and Kaley G. EDRF release from microvascular endothelial cells dilates arterioles *in vivo*. *Am. J. Physiol.* 261:H128-H133, 1991.

35. Moatter T and **Gerritsen, M.E.** Fibroblast growth factor upregulates PGG/H synthase in rabbit microvascular endothelial cells by a glucocorticoid independent mechanism. *J. Cell. Physiol.* 151:571-578, 1992.
36. Gundel, R.H., **Gerritsen, M.E.**, and Wegner, C.D. Polymyxin B-induced bronchial neutrophilia does not alter airway responsiveness to methacholine in cynomolgous monkeys. *Clin. Exp. Allergy* 22:357-363, 1992.
37. Carley, W.W., Niedbala, M.J. and **Gerritsen, M.E.** Isolation and partial characterization of human lung microvessel endothelial cells. *Am. J. Resp. Cell. Mol. Biol.* 7:620-630. 1992.
38. **Gerritsen, M.E.** and Bloor, C. Endothelial gene regulation in response to injury. *FASEB. J.* 7:523-532, 1993.
39. Nishida, M., Carley, W.W., **Gerritsen, M.E.**, Ellingsen, O., Kelley, R. and Smith, T.W. Isolation and characterization of human and rat cardiac microvascular endothelial cells and co-culture with adult rat ventricular myocytes. *Am. J. Physiol.* 264:H639-H652, 1993.
40. **Gerritsen, M.E.**, Szczepanski, A., Perry, C.A., Shen, C.P. Kelley, K.A., Ligon, G. and Carley, W.W. Regulation of the expression of intercellular adhesion molecule-1 (ICAM-1) in cultured human endothelial cells derived from rheumatoid synovium. *Arth. Rheum* 36:593-602, 1993.
41. Mannix, R.J., Kelley, K.A. and **Gerritsen, M.E.** A novel nucleotide receptor in rabbit microvascular endothelial cells. *Am. J. Physiology* 265:H675-680, 1993.
42. Acevedo, A.D., Bowser, S., **Gerritsen, M.E.** and Bizios, R. Morphological and proliferative responses of endothelial cells to hydrostatic pressure: role of fibroblast growth factor *J. Cell. Physiol.* 157:603-614, 1993
43. **Gerritsen, M.E.**, Niedbala M.J. and Carley, W.W. Cytokine regulation of microvascular endothelial cells. *Blood Cells* 19:325-342, 1993.
44. Szczepanski, A., Moatter, T.M., Carley, W.W. and **Gerritsen, M.E.** Induction of cyclooxygenase II in human synovial microvessel endothelial cells by IL-1. Inhibition by glucocorticoids *Arth. Rheum.* 37:495-503, 1994.
45. Kauffmann, D.J.H., van Meurs, J.C., Mertens, D.A.E., Peperkamp, E. and **Gerritsen, M.E.** IL-6 is elevated in vitreous of patients with proliferative vitreoretinopathy. *Invest Ophthal Vis Sci.* Vol 35:900-906, 1994.
46. Brown, Z., **Gerritsen M.E.**, Carley W.W., Streiter, R., Kunkel S.L., and Westwick J. Cytokine regulation of IL-8 and MCP-1 synthesis and release in human lung microvessel endothelium. *Am J. Pathol.* 145:913-921, 1994.

47. Moatter, T. and **Gerritsen, M.E.** FGF upregulates cyclooxygenase II in microvessel endothelium: role of protein kinase C. *Microcirculation* 1:79-88, 1994.
48. Shen, C.P. Ranges, G., Moatter, T., Ligon, G.F., Huwiler, K. Phan, S., Carley, W.W. and **Gerritsen, M.E.** Role of the p55 and p75 TNF receptors in the upregulation of ICAM-1 expression in synovial microvascular endothelium and synergistic actions of interferon gamma
49. **Gerritsen, M.E.**, Shen C.P., McHugh, M., Atkinson W.C., Kiely J., Milstone D., Luscinskas F.W. and Gimbrone M.A. Jr. Activation-dependent isolation and culture of murine pulmonary microvascular endothelium. *Microcirculation* 2: 151-163, 1995.
50. **Gerritsen M.E.**, Carley W.W., Ranges G.E., Shen C.P., Phan S.A. Ligon G.F. and Perry C.A. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am J Pathol* 147:1-15, 1995.
51. Abraham N.G., Lavrovsky, Y., Schwartzman M.L., Stoltz R.A., Levere, R.D., **Gerritsen M.E.**, Shibahara S and Kappas A. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. *Proc Natl Acad Sci (USA)* 92: 6797-6802, 1995
52. Stolz R.A., Conner M.S., **Gerritsen M.E.**, Abraham N.G., and Lanaiido-Schwartzman M. Direct stimulation of limbal microvessel endothelial cell proliferation and capillary formation in vitro by a corneal derived eicosanoid. *Am J Pathol.* 148:129-139, 1996.
53. Kalfa T.A., **Gerritsen M.E.**, Carlson E.C., Binstock A.J., and Tsilibary EC. Altered proliferation of retinal microvascular cells on glycated matrix. *Invest Ophthalmol Vis Sci* 36:2358-2367, 1996.
54. Chilian W.M., Bassingthwaighte J.B., Curry F.R., Cannon R.O., Davis M.J., Dellsperger K.C., Duling B.R., Fuster V., **Gerritsen M.E.**, Hoffman J.I.E., Kajiya F., Ku D.D., Lamping K.G., Laughlin H., Taylor A., and Small A.E.. The Coronary Microcirculation in Health and Disease. A NIH Workshop on the Coronary Circulation. *Circulation* 95:522-528, 1997.
55. Gundel R., Lindell D., Harris P., Fournel M., Jesmok G. and **Gerritsen M.E.** IL-4 induced leukocyte trafficking in cynomologous monkeys. Correlation with expression of adhesion molecules and chemokine generation *Clin Exp Allergy* 26:719-729, 1996.
56. Hoyt D.G., Mannix R.J., **Gerritsen M.E.**, Watkins S.A., Lazo J.S. and Pitt B.R. Integrins inhibit LPS-induced DNA strand breakage in cultured lung endothelial cells. *Am J Physiol* 14:L689-L694, 1996.
57. **Gerritsen, M.E.** Physiological and pathophysiological roles of eicosanoids in the microcirculation *Cardiovascular Res.* 32:720-732, 1996

58. Luscinskas, F.W. Ding H., Tan P., Cumming D., Tedder T.F. and **Gerritsen M.E.** L-selectin preferentially mediates monocyte attachment and rolling on TNF α activated vascular endothelium under flow in vitro. *J. Immunol.* 156:326-335, 1996
59. Medow M.S., Kletter L.B. and **Gerritsen M.E.** Glucocorticoid induced alterations in membrane fluidity: Evidence for membrane stabilizing effects mediated by glucocorticoid induced modifications of membrane lipid composition (submitted for publication)
60. Read, M, Neish A.S., **Gerritsen M.E.**, and Collins T. Nuclear I κ B α and the post-induction transcriptional repression of E-selectin and VCAM-1. *J. Immunol.* 157: 3472-3479, 1996
61. Panes, J, **Gerritsen, M.E.**, Anderson D.C., Miyasaka M. and Granger DN. Apigenin inhibits TNF-induced ICAM-1 upregulation in vivo. *Microcirculation* 3: 279-286, 1996
62. Carley W.W., Szczepanski A. and **Gerritsen M.E.** Human synovial endothelial cells co-express cytokeratins and hyaluronic acid: regulation of hyaluronic acid production by cytokines and fibroblast growth factor. *Microcirculation* 3: 359-370, 1996.
63. **Gerritsen M.E.**, Padgett R., Atkinson W.C., Gimbrone M.A. Jr, and Milstone, D. Endothelial cells from E-selectin deficient mice form tubes in vitro *Lab. Invest.* 175-184, 1996.
64. Schwartz, E., Bizios, R. Moatter, T. and **Gerritsen, M.E.** Endothelial gene regulation in response to hydrostatic pressure. (submitted for publication)
65. Barkalow F.J., Goodman M.J., **Gerritsen M.E.** and Mayadas T.N. Importance of P-selectin for neutrophil adhesion to microvascular endothelial cells of the brain. *Blood.* 88: 4585-4593, 1996.
66. **Gerritsen, M.E.**, Williams, A.J., Neish, A.S., Moore, S., Shi, Y and Collins, T. CBP/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci U S A* 94:2927-2932, 1997
67. Allport, J.R., Ding, H., Collins, T., Vestweber, D., **Gerritsen, M.E.** and Luscinskas, F.S. Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the VE-Cadherin complex at endothelial junctions. *J Exp Med* 186:517-527, 1997
68. Henninger, D.D., Panes, J., Russel, J., **Gerritsen, M.E.**, Anderson, D.C. and Granger, D.N. Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *J Immunol.* 158: 1825-1832, 1997
69. Pierce, J.W., Schoenleber, R., Jesmok, G., Best, J., Moore, S.A., Collins, T. and **Gerritsen, M.E.** Novel inhibitors of cytokine-induced I κ B α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem*; 272 : 21096-21103, 1997

70. Schwartz, E.A., Bizios, R., Medow, M.S., **Gerritsen, M.E.** Exposure of human vascular endothelial cells to sustained hydrostatic pressure stimulates proliferation. Involvement of the α integrins. *Circ. Res.* 84:315-322, 1999.
71. Komatsu, S., Flores, S., **Gerritsen, M.E.**, Anderson, D.C. and Granger, D.N. Differential upregulation of circulating soluble and membrane-bound forms of intercellular adhesion molecule-1. *Am J Pathol* 151:205-214, 1997.
72. Horie Y., Chervenak R.P., Wolf R., **Gerritsen M.E.**, Anderson D.C., Komatsu S., Granger D.N. Lymphocytes mediate TNF-alpha-induced endothelial cell adhesion molecule expression: studies on SCID and RAG-1 mutant mice. *J. Immunol* 159: 5053-5062, 1997
73. Hoyt D.G., Rizzo M., **Gerritsen M.E.** Pitt B.R., Lazo J.S. Integrin activation protects pulmonary endothelial cells from the genotoxic effects of bleomycin. *Am J Physiol* 273: L612-L617, 1997.
74. Henninger D.D., **Gerritsen M.E.**, Granger D.N. Low-density lipoprotein receptor knockout mice exhibit exaggerated microvascular responses to inflammatory stimuli. *Circ Res* 81:274-281, 1997.
75. **Gerritsen M.E.**, Shen C.P., Perry C.A. Synovial fibroblasts and the sphingomyelinase pathway: sphingomyelin turnover and ceramide generation are not signaling mechanisms for the actions of tumor necrosis factor-alpha. *Am J Pathol* 152:505-512, 1998.
76. Carley, W., Ligon, G., Phan, S., Dzuiba, J., Kelley, K., Perry, C.A. and **Gerritsen, M.E.** Distinct ICAM-1 forms and expression pathways in synovial microvascular endothelial cells. *Cellular and Molecular Biology* 45:79-88, 1999.
77. Sheppard, K.A., Phelps, K.M., Williams, A.J., Thanos, D., Glass, C.K., **Gerritsen, M.E.** and Collins, T. Nuclear integration of glucocorticoid receptor and NF-KB signaling by CREB binding protein and steroid receptor coactivator-1. *J. Biol. Chem.* 273:29291-29294, 1998.
78. Langley, R.R., Russell, J., **Gerritsen, M.E.**, Specion, R.D. and Granger, D.N. Quantification of murine endothelial cell adhesion molecules in solid tumors. *Am J. Physiol* 277:H1156-66, 1999.
79. Mori, N., Horie, Y., **Gerritsen, M.E.** and Granger D.N. Ischemia-reperfusion induced microvascular responses in LDL-receptor -/- mice. *Am J Physiol* 276:H1647-H165, 1999.
80. Yang, S., Graham, J., Kahn, J., Schwartz, E.A. and **Gerritsen, M.E.** Differential roles for CD31 and VE-cadherin in formation of vascular tubes and lumens in three-dimensional gels. *Am J. Pathol* 155:887-95, 1999.

81. Mori, N., Horie, Y., **Gerritsen, M.E.**, Anderson, D.C.. and Granger, D.N. Anti-inflammatory drugs and endothelial cell adhesion molecule expression in murine vascular beds. Gut 33:186-195, 1999.
82. Xin, X., Yang, S., Kahn, J., Kowalski, J. and **Gerritsen, M.E.** Peroxisome proliferator-activated receptor γ (PPAR γ) ligands are potent inhibitors of angiogenesis in vitro and in vivo. J. Biol. Chem. 274:9116-9121, 1999.
83. Eppihimer, M.J., Russell, J., Langley, R., **Gerritsen, M.**, Granger D.N. Role of tumor necrosis factor and interferon gamma in endotoxin-induced E-selectin expression. Shock 11:93-7, 1999.
84. Kahn, J., Mehraban, F., Ingle, G., Xin, X., Bryant, J.E., Vehar G., Schoenfeld J., Grimaldi C.J., Peale F., Draksharapu A., Lewin D.A., **Gerritsen M.E.**. Gene expression profiling in an in vitro model of angiogenesis. Am J Pathol 156:1887-900, 2000.
85. Li, B., Fuh, G., Meng, G., Xin, X., **Gerritsen, M.E.**, Cunningham, B., de Vos, A. Receptor-selective variants of human VEGF: generation and characterization . J. Biol. Chem.275(38):29823-8, 2000.
86. Peale, F.. and **Gerritsen, M.E.** Gene expression analysis in angiogenesis and vascular development. J. Pathol. 195:7-19, 2001.
87. Xin, X., Yang, Y., Ingle, G. , Zlot, C., Rangell, L., Kowalski, J. Schwall, R., Ferrara, N., and **Gerritsen, M.E.** Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo Am J. Pathol 158:1111-1120, 2000
88. Langley RR, Carlisle R, Ma L, Specian RD, **Gerritsen M.E.**, Granger DN. Endothelial expression of vascular cell adhesion molecule-1 correlates with metastatic pattern in spontaneous melanoma. Microcirculation 8:335-345, 2001.
89. Yang, S., Xin, X., Zlot, C., Ingle, G., Fuh, G., Li, B., Moffat, B., de Vos, A., and **Gerritsen, M.E.** Vascular Endothelial Cell Growth Factor-Driven Endothelial Tube Formation Is Mediated by Vascular Endothelial Cell Growth Factor Receptor-2, a Kinase Insert Domain-Containing Receptor .Arterioscler Thromb Vasc Biol 21:1934-1940, 2001
90. Shin, H. Bizios, R and **Gerritsen, M.E.**. Cyclic pressure modulates endothelial cell proliferation and apoptosis. Biomed. Eng. 30:. 296-304
91. Kiosses, W.B., Hood, J., Yang, S., **Gerritsen, M.E.**, Cheresch, D.A, Alderson, N. and Schwartz, M.A.. A dominant negative p65 PAK peptide inhibits angiogenesis. Circ, Res.90:697-702,, 2002
92. Schwartz, E.A., Shin, H., Bizios, R. and **Gerritsen, M.E.**. Role of the FGF receptor bek in hydrostatic pressure induced endothelial cell proliferation (submitted).

93. **Gerritsen, M.E.** Angiogenesis and Chronic Disease. Trends in Molecular Medicine 7: 333-334, 2001
94. **Gerritsen, M.E.**, Soriano, R., Zlot, C., Yang, S., Ingle, G., Toy, K. Peale, F., Wu, T. and Williams, P.M. In silico data filtering to identify new angiogenesis targets from a large in vitro gene profiling set. Physiological Genomics 10:13-20, 2002
95. Shin, H. , Bizios, R., and **Gerritsen, M.E.** Cyclic pressure modulates endothelial permeability and endothelial junctional proteins. Endothelium (in press)
96. **Gerritsen, M.E.** Peale, F. and Wu, T. Gene Expression Profiling in silico: Relative Expression of Candidate Angiogenesis Associated Genes in Renal Cell Carcinomas. Exp. Nephrology 10:114-119, 2002.
97. Zlot, C., Ingle, G., Hongo, J., Yang, S., Peale, F.V. and **Gerritsen M.E.** Stanniocalcin 1, a secreted glycoprotein focally expressed at sites of physiological and pathological angiogenesis, is an inhibitor of hepatocyte growth factor induced endothelial migration (J. Biol. Chem. accepted for publication)
98. Filvaroff, E., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., Hoeffel, J., Bunting S., Ross, J., Carano, R.A.D., Powell-Braxton, L., Wagner, G.F., **Gerritsen, M.E.** and French, DM., Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. Endocrinology 143:3681-90, 2002
99. Tejada, M., Pelletier, N. and **Gerritsen, M.E.**, A novel neuropeptide is a modulator of angiogenesis and permeability in vitro and in vivo (in preparation)
100. Yang, S. Toy, K., Ingle, G., Williams, PM, Fuh, G, Li, B, de Vos, A, and **Gerritsen, M.E.**, Vascular endothelial growth factor-induced genes in human umbilical vein endothelial cells: relative roles of KDR and Flt-1 receptors. Arterioscler. Thromb. Vasc. Biol. 22:1797-803, 2002
101. Paoni, N.F., Peale, F., Wang, F., Errett-Baroncini, C., Steinmetz, H., Toy, K., Bai, W., Williams, P.M., Bunting, S., **Gerritsen, M.E.**, and Powell-Braxton, L. Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice. Physiological Genomics 11:262-72. 2002
102. Yang, S. Lee, B.J., DeVaux, B. and **Gerritsen M.E.** 19C11, an endothelial specific antibody, selectively recognizes an antigen on proliferating endothelial cells (in preparation).
103. Shin, H., Smith, M.L., Toy, K., Williams, P.M. and **Gerritsen, M.E.**, VEGFC mediates cyclic pressure induced endothelial cell proliferation. Physiological Genomics 11:245-51, 2002
104. Chang, T-L, **Gerritsen, M.E.**, Hill, M. Kubes, P. and Perry, M., Microcirculation down under. Trends in Pharmacological Science 23:106-8, 2002.

105. **Gerritsen, M.E.**, Soriano, R, Yang, S, Zlot, C, Ingle, G, Toy, K and Williams, P.M.. Branching Out: A molecular fingerprint of endothelial differentiation into tube-like structures generated by Affymetrix oligonucleotide arrays. *Microcirculation* 10: 63-81, 2003
106. Eggington, S. and **Gerritsen, M.** Lumen formation: in vivo versus in vitro observations. *Microcirculation* 10:45-61, 2003.
108. Yang, RB, Ng, CKD, Wasserman, SM, Komuves, LG, **Gerritsen, ME** and Topper, JN. A novel IL-17 receptor like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. *J. Biol. Chem.* 278:33232-8,2003
109. **Gerritsen, ME**, Tomlinson, JE, Zlot, C, Ziman, M, Hwang, S. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br. J. Pharmacol* 140:595-610, 2003
110. Zlot, C. Ingle, G, Hong, J. Yang, S, Shen, Z, Schwall, R, Paoni, N, Wang, F, Peale, FV jr, **Gerritsen, ME.** Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. *J Biol Chem.* 278: 47654-9.
111. Shin, HY, Bizios, R, **Gerritsen, ME.** Cyclic pressure modulates endothelial barrier function. *Endothelium* 10:179-187, 2003.
112. Gerritsen, M.E. Genetic variations in vascular endothelial growth factor and endothelial nitric oxide synthase and their contributions to vascular disease. *Microcirculation* (in press)
113. Gerritsen, M.E. HGF and VEGF-Dynamic Duo. *Circulation* (in press)

Book chapters

1. **Gerritsen, M.E.** and Lederis, K. Effects of urotensin I on isolated blood vessels. *Proc. West. Pharmacol. Soc.* 19:461-465, 1976.
2. **Gerritsen, M.E.** Mathison, R. and Lederis, K. Urotensin I induced relaxation and intracellular cAMP. *Proc. West. Pharmacol. Soc.* 20:133-137, 1977
3. **Gerritsen, M.E.** and Lederis, K. Effects of urotensin I on the isolated rat tail artery: possible mediation of the relaxation response by cAMP. *Proc. West. Pharmacol. Soc.* 21:250-253, 1978.

4. Parks, T.P., **Gerritsen, M.E.**, Printz, M.P. and Lederis K. Prostaglandin production in the rat tail artery and vein. *Proc. West. Pharmacol. Soc.* 21:241-244, 1978.
5. **Gerritsen, M.E.** Vascular actions of urotensin I. Doctoral Dissertation. University of Calgary, 1978.
- 6.. **Gerritsen, M.E.**, Weinstein, B.I. and Southren, A.L. Arachidonate metabolism and prostaglandin release from human trabecular meshwork cells. In: Prostaglandins, Leukotrienes and Lipoxins. J. Martin Bailey, Ed. Plenum Press 185-192, 1985.
7. **Gerritsen, M.E.**, Milici, A.J. and Carley, W.W. Microvascular endothelial cells: isolation, identification and culture. Advances in Cell Culture 6:35-37, 1988.
8. **Gerritsen, M.E.** and Medow, M.S. A role for extracellular sodium in the regulation of microvascular endothelial prostaglandin production. In Microcirculation, an update. (Tsuchiya, M., Asano, M., Mishima, Y. and Oda, M. eds)735-738, 1988.
9. **Gerritsen, M.E.** and Mannix, R.J. G-proteins and phospholipase activation in endothelial cells. Adv. Exp. Med. Biol. Phospholipase A₂, role and function in Inflammation. P.Y.K. Wong and E. Dennis, Eds. Vol 275: 115-124, 1990.
10. Szczepanski, A., Carley, W.W., Marshall, P. Mullins, L., Rayan, G. and **Gerritsen, M.E.** Isolation and characterization of synovial endothelial cells from patients with rheumatoid arthritis. Eicosanoid production. In: Advances in Rheumatology and Inflammation. 2. Leukotrienes and the vascular phenomena of Inflammation related to rheumatoid arthritis. (EULAR, Babel). R. Muller Peddinghaus, ed 119-128, 1992.
11. Capetandes, A. and **Gerritsen, M.E.** Heterogeneity in retinal microvascular endothelial cell growth control by pericytes. In Biology of the Ocular Microcirculation (Weinreb, R.N. and L.A. Wheeler, eds. Elsevier) 81-90, 1992.
12. Kalfa, T.A. **Gerritsen, M.E.**, Tsilibary, E.C. Altered proliferation of retinal microvascular cells in response to non-enzymatic glycosylated matrix proteins. In Angiogenesis: Molecular Biology, Clinical Aspects, M.E. Marangoudakis (ed). Plenum Publishing Company, London. pp 101-108.
13. **Gerritsen, M.E.** Physiological Functions of Endothelium. Chapter 1 In Vascular Medicine (2 ed).(J Loscalzo, MA Creager and VJ Dzau eds. Little Brown and Company, Boston MA USA) pp 3-39.
14. Silverman E.S., **Gerritsen, M.E.** and Collins T. Metabolic Functions of the Pulmonary Endothelium. Chapter 43 in THE LUNG: Scientific Foundations (Second Edition) RG Crystal, JB West, E Weibel and P Barnes ed . Lippincott-Raven, Philadelphia. pp 629-651

15. **Gerritsen, M.E.** Targeting Transcriptional Regulation of Adhesion Protein Expression: A new approach to anti-adhesion therapy. In Collins, T. Transcriptional Regulation of Adhesion Protein Expression (in press)
16. **Gerritsen, M.E.** Flavonoids: Inhibitors of cytokine induced gene expression. In: Flavonoids in the Living System, Adv Exp Med Biol.439. Manthey and Buslig eds. Plenum Press, New York, 1998. pp 183-190.
17. Schwartz, EA, Bizios, R, and **Gerritsen, M.E.** Effects of sustained hydrostatic pressure on the expression of endothelial cell-leukocyte adhesion molecules. In: Pulmonary Edema, Wier, EK and Reeves, JT (eds) Future Publishing Co., Armonk NY 1998. pp 195-203.
18. Schwartz, EA, Bizios, R and **Gerritsen, M.E.** Effects of hydrostatic pressure on endothelial cells. In: Physical Forces and Vascular Cells. PE. Lelkes, Editor. Harwood Academic Publishers Amsterdam 1999 pp275-290.
19. Collins, TC and **Gerritsen, M.E.** Preface: transcriptional control of cell adhesion molecules. In: Leukocyte recruitment, endothelial cell adhesion molecules and transcriptional control. Insights for drug discovery. T Collins, Editor Kluwer Academic Publishers, Boston, 2000
20. Parks, TP and **Gerritsen, M.E.** Regulation of Intercellular Adhesion Molecule (ICAM) Gene Expression. In: Leukocyte recruitment, endothelial cell adhesion molecules and transcriptional control. Insights for drug discovery. T Collins, Editor Kluwer Academic Publishers, Boston, 2000. pp 109-174.
21. **Gerritsen, M.E.** . Therapeutic regulation of leukocyte adhesion molecule expression. . In: Leukocyte recruitment, endothelial cell adhesion molecules and transcriptional control. Insights for drug discovery. T Collins, Editor Kluwer Academic Publishers, Boston, 2000. pp 263-303.
22. **Gerritsen, ME.** Endothelial Heterogeneity. In. Encyclopedia of the Microvasculature Academic Press, (in press, 2004)
23. **Gerritsen, ME.** Gene profiling techniques and their application in cardiovascular disease. In Cardiovascular Genomics, MK Raizada ed. Human Press. (in press)
24. **Gerritsen, ME.** Endothelial phenotypes: genomic diversity. In. Endothelial phenotypes in health and disease. W. Aird ed. (in preparation).
25. **Gerritsen, ME.** Angiogenesis. In: Handbook of Physiology (in press)
26. **Gerritsen, ME** and Wagner, G. Stanniocalcin-No longer just a fish tale. In: Vitamins and Hormones, Elsevier Press. (in press)
27. **Gerritsen, ME.** Therapeutic Angiogenesis. Encyclopedia of Molecular Medicine (in press)

28. Gerritsen, ME and Parsons, R Masdevallias, gems of the orchid world. Timber Press (in press)

Patents: Issued or Published.

(note that there I have over 900 patent applications pending; for the sake of brevity a few representative ones are listed)

WO0104311 Secreted and Transmembrane Polypeptides and Nucleic Acids

WO9914234 PROMOTION OR INHIBITION OF ANGIOGENESIS AND CARDIOVASCULARIZATION

WO0030628 METHOD OF INHIBITING ANGIOGENESIS

WO0103720 CARDIOVASCULAR USES FOR GLITTER/GITR

WO0125433 ANGIOGENESIS MODULATING GENES

WO0032776 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

WO0053756 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

WO0073454 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

WO0053757 PROMOTION OR INHIBITION OF ANGIOGENESIS AND CARDIOVASCULARIZATION

WO0073445 PROMOTION OR INHIBITION OF ANGIOGENESIS AND CARDIOVASCULARIZATION

WO0077037 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

WO0116318 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

WO0140466 SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME

A Novel Calcium Signaling Pathway Targets the *c-fos* Intragenic Transcriptional Pausing Site*

(Received for publication, June 15, 1999, and in revised form, July 29, 1999)

Vincent Coulon†§, Jean-Luc Veyrune†¶, Nikolai Tourkine, Annick Vié, Robert A. Hipkind, and Jean-Marie Blanchard||

From the Institut de Génétique Moléculaire, CNRS, UMR 5535, IFR24, 1919 route de Mende, 34293 Montpellier cedex 5, France

In many cell types, increased intracellular calcium gives rise to a robust induction of *c-fos* gene expression. Here we show that in mouse Ltk⁻ fibroblasts, calcium ionophore acts in synergy with either cAMP or PMA to strongly induce the endogenous *c-fos* gene. Run-on analysis shows that this corresponds to a substantial increase in active polymerases on downstream gene sequences, i.e. relief of an elongation block by calcium. Correspondingly a chimeric gene, in which the human metallothionein promoter is fused to the *fos* gene, is strongly induced by ionophore alone, unlike a *c-fos* promoter/ β -globin coding unit chimeric construct. Internal deletions in the hMT-*fos* reporter localize the intragenic calcium regulatory element to the 5' portion of intron 1, thereby confirming and extending previous *in vitro* mapping data. Ionophore induced cAMP response element-binding protein phosphorylation on Ser¹³³ without affecting the extracellular signal-regulated kinase cascade. Surprisingly, induction involved neither CaM-Ks nor calcineurin, while the calmodulin antagonist W7 activated *c-fos* transcription on its own. These data suggest that a novel calcium signaling pathway mediates intragenic regulation of *c-fos* expression via suppression of a transcriptional pause site.

The proto-oncogene *c-fos* represents the prototype for the family of immediate early genes. Its activation follows stimulation of the cell by a wide range of extracellular stimuli but is independent of protein neosynthesis (1–3). *c-fos* expression is regulated at multiple levels by intracellular signaling events acting in synergy (for reviews, see Refs. 4–7). The majority of studies on how signal transduction cascades modulate *c-fos* gene expression have focused on its upstream promoter sequences. Several cis-acting elements present in this region have been characterized as targets for numerous stimuli (8–11): the *v-sis* inducible element (12, 13), the serum response element (SRE)¹ (7, 14–16), the Fos AP1-like site (16) and the cAMP response element (CRE) (17–21).

* This work was supported in part by grants from the CNRS, BIOMED2 program, Association pour la Recherche contre le Cancer (ARC), and the Ligue Nationale contre le Cancer (to J. M. B.) and the Fondation pour la Recherche Médicale and ARC (to R. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Contributed equally to the results of this work.

§ Supported by a grant from the French Ministry for Education and Research.

¶ Supported by a contract from the BIOMED2 program.

|| To whom correspondence should be addressed. Tel.: 33-4-67-61-36-49; Fax: 33-4-67-04-02-31; E-mail: blanchard@igm.cnrs-mop.fr.

¹ The abbreviations used are: SRE, serum response element; CRE, cAMP response element; CREB, cAMP response element-binding pro-

In several cell types calcium mobilization plays a central role in the modulation of *c-fos* gene expression; however, the mechanisms involved are still not fully understood. Calcium ions act as intracellular secondary messengers either after entering cells through various ion channels and/or upon release from internal stores. Ca²⁺ differentially activates cellular processes, and immediate early genes such as *c-fos* gene provide important targets to characterize how the calcium signal is transduced to the nucleus to activate various transcription programs (22, 23). Based on results from mutagenesis and transient transfection analyses, calcium has been proposed to activate a variety of pathways targeting different promoter elements (8, 21). Some are mediated by SRE-dependent processes. In some instances this occurs via the well characterized Ras-Raf-Erk-Elk-1 signaling module (23–26). In other situations increased levels of intracellular calcium induced by membrane depolarization with elevated levels of KCl or exposure to the calcium ionophore ionomycin have been shown to activate the *c-fos* promoter in PC12 pheochromocytoma cells. These have been linked to SRF independently of Elk-1 (24). SRF-driven activation did not involve Ras but did appear to involve calcium/calmodulin-dependent kinases (25). The mechanism is still uncertain, since mutation of the major phosphorylation site in SRF showed continued activity in this study. Finally some evidence implies that calcium signals to the Fos AP1-like element immediately downstream of the SRE-binding site (26), even though no transcription factor has been directly implicated in control via the Fos AP1-like site alone.

Other pathways activate *c-fos* transcription independently of the SRE, primarily via the CRE located at position –65 (reviewed in Refs. 27 and 28). This element is sufficient to mediate calcium-dependent reporter gene activation in some cell contexts, while in other cells additional cryptic CREs in the upstream promoter contribute to reporter gene activity (19). To further complicate the role of the CRE, intracellular calcium fluxes can also activate kinases downstream of Ras and ERK that phosphorylate CREB at serine 133 and thus potentially modulate transcription through the CRE (14, 29).

An explanation for the multiplicity of the effects mediated by calcium on gene expression has recently been provided by elegant microinjection experiments aimed at unraveling how spatially distinct calcium signals generate diverse transcriptional responses (30). Nuclear injection of a non-diffusible calcium chelator blocked increases in nuclear, but not cytoplasmic, calcium concentrations following activation of L-type voltage-

tein; DRE, downstream regulatory element; IBMX, isobutylmethylxanthine; PMA, phorbol 12-myristate 13-acetate; 8-Br-cAMP, 8-bromo-cAMP; ERK, extracellular signal-regulated kinase; CaM, calmodulin; CaM-K, CaM-activated kinase family; SRF, serum response factor; MOPS, 4-morpholinopropanesulfonic acid; MTLIIa, human metallothionein IIa promoter; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

gated calcium channels in a mouse pituitary cell line. Using reporters driven by different *c-fos* promoter regions, Hardingham *et al.* (30) showed that increases in nuclear calcium control CRE-mediated transcription, whereas a rise of cytoplasmic calcium activated SRE-driven transcription. In fact, this suggests that the mode of calcium entry and the cell type determine which upstream promoter element is required for the activation of a transiently introduced reporter gene. Accordingly the CRE alone can mediate activation by calcium signals triggered by membrane depolarization of PC12 pheochromocytoma cells (21, 22, 31), an effect that is not reproduced in HeLa cells (8).

More recently, a new calcium-sensitive transcriptional repressor has been proposed to bind a downstream regulatory element (DRE) present within the human prodynorphin gene (32). Upon stimulation by calcium this repressor, named DREAM for DRE-antagonist modulator, is no longer able to bind the DRE. In addition to prodynorphin promoter, DREAM represses also transcription from the *c-fos* gene in a transient transfection assay. However, whether this is true for the endogenous gene remains to be established.

A close inspection of the *c-fos* transcription unit through high resolution run-on analysis has also suggested the involvement of intragenic regulatory elements as important targets of *c-fos* regulation by calcium (33–36). In cultured macrophages, *c-fos* transcription is stimulated by multiple pathways requiring the mobilization of calcium from internal sources (34, 37). A strong block to transcriptional elongation, mapping beyond *c-fos* exon 1, was observed when freshly isolated peritoneal macrophages were put into primary culture (34). Calcium-dependent relief of this block strongly increased *c-fos* mRNA levels. In T cells, elevated cytoplasmic calcium is a critical mediator of activation upon stimulation of the antigen receptor. The synergistic action of calcium ionophore and agonists of protein kinase C mimics authentic antigen treatment in some T cell hybridomas (38). In the latter case, the principal effect of calcium was shown to be on the elongation of *c-fos* transcripts (35).

Using nuclear extracts from Ltk⁻ cells, we had previously mapped an *in vitro* arrest site within the murine *c-fos* gene (39). In this work we confirm and extend these results *in vivo*. However, because most previous studies on *c-fos* transcription have dealt with transient transfection experiments, we sought to use permanent cell lines carrying integrated reporter genes. We find that a sequence within *c-fos* intron 1, while barely active on its own, can strongly augment a calcium ionophore-driven transcriptional response together with its homologous or a heterologous upstream promoter. Even though this is correlated with CREB phosphorylation on Ser¹³³, it is mediated by a novel signaling pathway that surprisingly is potentiated by the calmodulin antagonist W7.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Tissue culture medium, penicillin, streptomycin, glutamine, G418, Random primers DNA labeling system, and TRIZOL were obtained from Life Technologies, Inc. (Cergy Pontoise, France). Phorbol myristate acetate (PMA), 3-isobutyl-1-methylxanthine (IBMX), 8-bromo cyclic adenosine monophosphate (8-Br-cAMP), A23187, W5, W7, KN62, KN93, and secondary antibodies were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Antisera directed against phosphorylated forms of CREB and ERK came from New England Biolabs (Ozyme, Paris), all radioactive nucleotides and ECL reagents were from Amersham Pharmacia Biotech, the pHOOK-2 vector and Capture-Tec beads came from Invitrogen (Groningen, The Netherlands), and all restriction enzymes and bovine serum albumin from Roche Molecular Biochemicals (Meylan, France). The calmodulin-dependent protein kinase assay kit was purchased from Upstate Biotechnology Inc. (EUROMEDEX, France). Bradford protein assay kit was from Bio-Rad (Ivry Sur Seine, France) and Immobilon polyvinylidene difluoride membranes from Millipore. Radioactive signals were revealed by autoradiography using intensifying screens at -80 °C and quantitated by PhosphorImager technology.

Cell Culture—Mouse Ltk⁻ fibroblasts were grown at 37 °C in a 5% CO₂ containing atmosphere in the presence of 10% fetal calf serum in Dulbecco's modified Eagle's medium. When indicated, cells were serum starved for 24 h and stimulated by refeeding with 10% serum for the indicated times. 3–5 × 10⁶ exponentially growing cells were treated with either 100 nM PMA, 100 mM IBMX + 1 mM 8-Br-cAMP, 0.1–10 μM rapamycin or cyclosporin A, 10 μM KN62 or KN93, 2.5–250 μM W5 or W7, 20 μM PD98059, or 10 mg/ml of the calcium ionophore A23187 alone, or a combination of these agents as indicated in the legends of the figures. All media were supplemented with streptomycin, penicillin, and L-glutamine. Cells were transfected with the various constructs described in the figures using calcium phosphate and pools of G418-resistant cells were used throughout this work.

pHOOK-2 Transient Transfection and Selection—6 μg of pHOOK-2-Lac Z (control), CaM-KIIa-HOOK, CaM-KIIa-HOOK, or CaM-KIVa-HOOK were transfected in 3–5 × 10⁶ Ltk⁻ p19/1 cells by the calcium phosphate technique. 16 h later, cells were washed with HS buffer (25 mM Hepes, 140 mM NaCl, pH 7.4), fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum was added and after 2 h A23187 stimulation was performed, where indicated, for 1 h. Cells were then detached with 6 mM EDTA, spun down, and resuspended in 5 ml of Dulbecco's modified Eagle's medium, 10% fetal calf serum, also containing A23187 where indicated. 50 μl of magnetic beads (Invitrogen Capture-Tec system) were added and tubes were gently stirred at 37 °C for 1 h. After selection on a magnetic stand and 3 washes with Dulbecco's modified Eagle's medium, 10% fetal calf serum, the cells were split into two batches: one was resuspended in TRIZOL reagent for RNA extraction while the other one was treated with Laemmli's polyacrylamide gel electrophoresis-SDS sample buffer.

Nuclear Run-on Transcription—Extraction of nuclei, run-on transcript labeling and hybridization were carried out as described (39). Preparations of crude nuclei were split into aliquots containing 5 × 10⁷ nuclei which were frozen in liquid nitrogen and thawed immediately prior to the labeling reaction. Incubations were carried out at 30 °C for 30 min in the presence of 100 μCi of [α-³²P]UTP (400 Ci/mmol, 10 μCi/μl). Labeled run-on transcripts were purified and hybridized to nitrocellulose filters containing equimolar amounts of the plasmids indicated. Hybridization was carried out for 48 h at 42 °C. Filters were washed twice at 65 °C in 0.2 × SSC and at 25 °C in 2 × SSC containing 2 μg/ml DNase-free RNase A. Signals were corrected for the thymidine content of each hybridizing DNA strand and standardized to those obtained with the *gapdh* cDNA probe.

RNA Blots and RNase Protection Assay—Total RNA was extracted using a standard 5 M guanidinium thiocyanate-phenol procedure at pH 5. Blots were sequentially hybridized to a mouse *c-fos* and *gapdh* cDNA probes labeled by random priming with [α-³²P]dCTP (3000 Ci/mmol). The RNA probe was prepared from PM37.37 (containing a mouse genomic *c-fos* DNA spanning nucleotides -599 to +251, cloned into pBluescript) linearized with *Bss*HII, uniformly labeled with [α-³²P]UTP (400 Ci/mmol), and purified by polyacrylamide gel electrophoresis. 20 μg of RNA for each sample was hybridized, processed for degradation by RNase A, and the resulting protected bands analyzed by electrophoresis in 5% polyacrylamide sequencing gels as described (40).

Plasmid Constructs—For *c-fos* constructs the starting construct was p19/1, which contains a 4-kilobase *Nae*I-*Bam*HI mouse genomic DNA fragment under the control of the human metallothionein IIa promoter (41). Large deletions were generated using unique *Xho*I, *Xba*I, and *Sal*I restriction sites and religation as indicated. Intron 1 small deletions were generated with exonuclease III on *Xho*I-linearized p19/1, followed by blunt-ending by S1 nuclease treatment and religation. Plasmids containing overlapping deletions were selected and sequenced. pMT-globin was derived from p19/1 after deleting the *Bam*HI-*Bam*HI *c-fos* fragment and replacing it with a rabbit β-globin genomic fragment. pFos-globin contained the rabbit β-globin gene under the control of the *c-fos* promoter contained in the *Sma*I-*Pvu*II DNA fragment spanning nucleotides -500 to -19 relative to the transcription initiation site (42).

The CaM kinase II catalytic subunit (amino acids 1–290) was derived from plasmids pSG5-192 I (inactive) or pSG5-192 A (active) (Ref. 43, kind gift from A. Means) by *Hind*III and *Bam*HI digestion. These fragments were cloned in the pHOOK-2 vector to produce CaM-KIIa-HOOK and CaM-KIIa-HOOK. The CaM kinase IV catalytic subunit (amino acids 1–313) was obtained from plasmid RSV-CaM-KIV (Ref. 44, kindly provided by S. Soderling) by *Hind*III and *Bgl*II digestion and cloned in pHOOK-2 vector to produce CaM-KIVa-HOOK.

Western Blots and Kinase Assays—5 × 10⁶ exponentially growing cells were treated with various inhibitors for 1 h prior to A23187 stimulation for 10 min as indicated and then lysed as described previously (45). Protein concentrations were determined using the Bradford

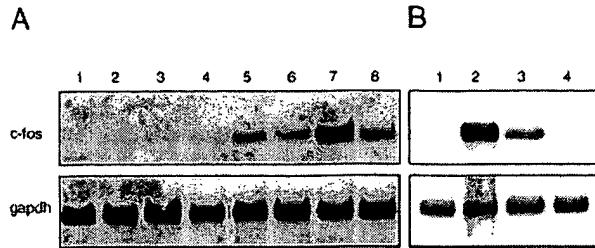


FIG. 1. Calcium ionophore is required for robust, sustained induction of *c-fos* by PMA or cAMP in Ltk⁻ cells. *A*, exponentially growing Ltk⁻ cells were left uninduced (lane 1) or stimulated for 1 h (lanes 2–6), 2 (lane 7), or 4 h (lane 8) with PMA (lane 2), IBMX + 8-Br-cAMP (lane 3), A23187 (lane 4), or a combination of A23187 and PMA (lane 5) or IBMX + 8-Br-cAMP (lanes 6–8). 20 μ g of total RNA was fractionated by electrophoresis through a formaldehyde-agarose gel, transferred onto nylon membranes and hybridized successively to *c-fos* (top panels) and *gapdh* (bottom panels) probes. *B*, transient expression of *c-fos* mRNA induced by serum restimulation of Ltk⁻ cells starved for 24 h. Total RNA was prepared from either serum-starved cells (lane 1) or cells restimulated for 30 (lane 2), 60 (lane 3), or 90 min (lane 4) and analyzed as above.

assay. 5 μ g of whole cell extracts were fractionated by electrophoresis through 8.5% SDS gels and transferred onto polyvinylidene difluoride membranes. After a quick dip into methanol, membranes were saturated for 1 h at room temperature in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin (fraction V). Primary antibodies (anti-phospho-Thr¹⁸³/Tyr¹⁸⁵ ERK or anti-phospho-Ser¹³³ CREB) were incubated overnight at 4 °C in the same medium at a 1/1000 dilution. After 3 washes with TBST, an anti-rabbit IgG antibody was added at a 1/5000 dilution for 1 h at room temperature. Antibody complexes were revealed by enhanced chemiluminescence (ECL kit) after 6 washes 5 min each with TBST.

CaM-activated kinases were purified through a small scale quick batch binding of a whole cell extract (100 μ g) to a calmodulin affinity resin (Stratagene). Activity was measured by incubating the immobilized kinases for 10 min at 30 °C in 50 μ l of a mixture containing 100 μ M auto Camtide II, 2 μ M each of PKC and PKA inhibitor peptides, 25 mM MgCl₂, 100 μ M [γ -³²P]ATP (10 μ Ci), 20 mM MOPS, pH 7.2, 25 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl₂. Incorporated radioactivity was monitored by liquid scintillation after spotting 25 μ l of reaction mixture onto P81 phosphocellulose paper and several washes with 0.75% phosphoric acid, according to the supplier's recommendations.

RESULTS

***c-fos* Induction in Ltk⁻ Cells Requires the Concerted Action of Calcium Ionophore and Phorbol Esters or cAMP**—We initially analyzed the response of mouse Ltk⁻ fibroblasts to agents that elevate intracellular levels of calcium and/or cAMP, since these pathways were shown to synergize in *c-fos* activation (46, 47). In Ltk⁻ cells, PMA, IBMX + 8-Br-cAMP, or calcium ionophore (A23187) generated only a minor induction of *c-fos* mRNA (Fig. 1A). In contrast, co-treatment with A23187 and either PMA or IBMX + 8-Br-cAMP gave rise to a much stronger induction of *c-fos* (Fig. 1A). Notably the signal was still high after 4 h of stimulation by a combination of A23187 + IBMX + 8-Br-cAMP, whereas it was undetectable 90 min after serum refeeding (Fig. 1B). This strong induction was sensitive to actinomycin D (data not shown; Fig. 4), indicating that it resulted from an increase in *de novo* transcription.

This was confirmed by a run-on analysis carried out on nuclei prepared from cells treated for 1 h with A23187, 8-Br-cAMP and IBMX (Fig. 2). The labeled nascent transcripts were hybridized to two DNA fragments spanning the 5'-half of the murine *c-fos* gene. The first one, (A), contains the first exon and the 3'-half of the first intron. The second (B), spans the 3'-half of the first intron, the second exon and the 5'-half of the second intron. Previous work has shown that *c-fos* transcription can be regulated in part at the level of elongation (34, 35, 40, 48, 49), and fragment A contains the premature termination site

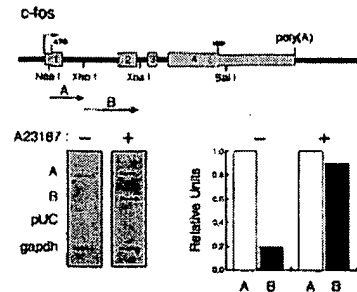


FIG. 2. Ionophore treatment relieves a transcriptional block within *c-fos* intron 1. Nuclei were isolated from exponentially growing Ltk⁻ cells stimulated for 1 h with IBMX + 8-Br-cAMP in the absence of A23187 (–) or presence (+). Nascent transcripts were labeled *in vitro* with [α -³²P]UTP and then hybridized to *c-fos* mouse genomic DNA fragments spanning nucleotides +42 to +580 (fragment A) and +580 to +1473 (fragment B) relative to the transcription start site. *c-fos* signals were normalized to *gapdh* signals after PhosphorImager quantitation and correction for the uridine content of each transcript. The main features of *c-fos* transcription unit are diagrammed: shaded rectangles represent exons, black bars correspond to intron or flanking sequences, the transcription initiation site is represented by a broken arrow, and the horizontal arrows below the gene show the *NaeI*-*XhoI* (fragment A) and *XhoI*-*XbaI* (fragment B) DNA fragments used for the run-on transcript hybridization. *Left panels*, autoradiograms of membranes hybridized to *in vitro* synthesized nuclear RNA from either untreated cells (–) or cells exposed to A23187 + IBMX + 8-Br-cAMP for 1 h (+). pUC and *gapdh* refer to pUC18 and rat *gapdh* cDNAs. *Right panel*, the hybridization signals on fragments A and B are represented relative to the signal on A, after normalization for their specific activity and the corresponding *gapdh* signal.

mapped *in vitro* (39). Prior to stimulation, a signal was detected on the promoter proximal fragment, whereas that from fragment B was disproportionately low, especially upon correction for the amount of uridine transcribed into RNA hybridizing to each fragment. Induction gave rise to a small increase in transcription of fragment A, together with a strongly enhanced signal from fragment B. Thus, in Ltk⁻ cells the endogenous *c-fos* gene is regulated at the level of transcriptional elongation.

Interestingly, A23187 also acts to stabilize *c-fos* mRNA. Cells were stimulated for 1 h with A23187 + IBMX + 8-Br-cAMP, and then actinomycin D was added for the indicated times (Fig. 3A). *c-fos* mRNA was still detectable 4 h after actinomycin D addition, whereas the same level was obtained with a 1-h actinomycin D chase after serum induction (Fig. 3B). The synergy between calcium ionophore and the other inducers suggests that both upstream and downstream regulatory elements are required for a full response to calcium in these cells.

***A c-fos* Gene under the Control of an Heterologous Promoter Shows a Full Response to Calcium Ionophore**—To address the contribution of regions downstream from the promoter to the calcium effect, we transfected Ltk⁻ cells with a construct expressing the mouse *c-fos* gene under the control of the human metallothionein IIa promoter (MTLIIa) (p19/1; Ref. 41), which has high basal activity. A23187 alone was sufficient to substantially induce (60-fold) the transfected gene (Fig. 4A), unlike the endogenous *c-fos* gene (see above). A slightly reduced induction (30-fold) was observed with a mutant lacking the 3'-untranslated region (p19/ Δ NsiI-MstII) that was previously shown to enhance mRNA stability (49–52). This is consistent with the increase in stability observed above for the endogenous gene. Actinomycin D blocked the induction but had a less striking effect on basal transcription (Fig. 4A). This observation was confirmed by RNase protection using an antisense probe spanning from nucleotides –95 to +251 relative to the transcription start site (Fig. 5A). The *c-fos* mRNA generated by the transfected gene lacks the first 42 nucleotides and can thus easily be distinguished from the endogenous mRNA (see "Experimental

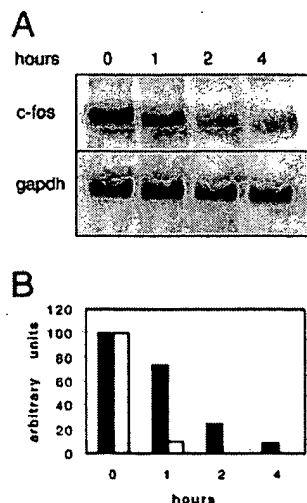


FIG. 3. Calcium ionophore leads to *c-fos* mRNA stabilization. *A*, Northern blot analysis of *c-fos* mRNA decay after treatment with A23187 + IBMX + cAMP for 1 h prior to addition of actinomycin D for the indicated times. RNA was processed as described in the legend of Fig. 1. *B*, quantitation of the data presented in panel *A* after normalization to the *gapdh* signals (closed bars). Also shown is the decay rate of *c-fos* mRNA following a 30-min serum induction (open bars).

Procedures"). As shown in Fig. 5*B*, the transfected gene was induced to the same level by calcium ionophore alone or together with IBMX + 8-Br-cAMP (lanes 6 and 7) or PMA (not shown), whereas the endogenous *c-fos* mRNA was observed only after combined treatment (lanes 4 and 7). The RNA samples used in this experiment are those used for the Northern blot of Fig. 1.

This effect was related to the *fos* gene, since no modulation by calcium was seen when a rabbit β -globin genomic sequence was placed downstream from the MTLIIa promoter (Fig. 4*B*, lanes 1 and 2). Nevertheless the same construct was inducible by Zn^{2+} (data not shown). When the same β -globin genomic sequence was appended 3' to the *c-fos* promoter (-400 to +10), the resulting globin mRNA was only modestly induced by calcium (at most 3-fold, Fig. 4*B*, lanes 3 and 4), in contrast to the strong induction of the complete *c-fos* gene (Fig. 4*A*). The *c-fos* promoter-globin coding construct was still inducible by serum (data not shown; Ref. 53). Since similar results were observed with other promoters linked to the *c-fos* gene (adenovirus MLP, rat β -actin; data not shown), we conclude that sequences located within the *c-fos* gene are required for a full transcriptional response to calcium.

The Intragenic Calcium Response Requires Sequences Located within *c-fos* Exon 1 and Intron 1—We then employed deletion mutagenesis to characterize the portion of the gene responsible for calcium sensitivity. A first series of deletions was generated using several restriction sites scattered along the transcription unit, as diagrammed in Fig. 6*A*. Plasmids corresponding to the various minigenes were transfected into Ltk⁻ cells, and pools of neomycin-resistant cells were used for monitoring A23187 inducibility. Deletions spanning sequences downstream of the middle of intron 1 were induced to approximately the same level as the starting p19/1 construct (35–55-fold, Δ XS and Δ XX, Fig. 6). Δ NX, which removes exon 1 and the 5' portion of intron 1, was only poorly induced (2–3-fold) and exhibited a high constitutive level of expression prior to stimulation. The region deleted in Δ NX contains a previously mapped *in vitro* arrest site (39), as well as an *in vivo* pause site (40). This suggests that calcium acts at the level of elongation rather than initiation. Consistent with this, *c-fos* mRNA induction by calcium ionophore in p19/1 stably transfected

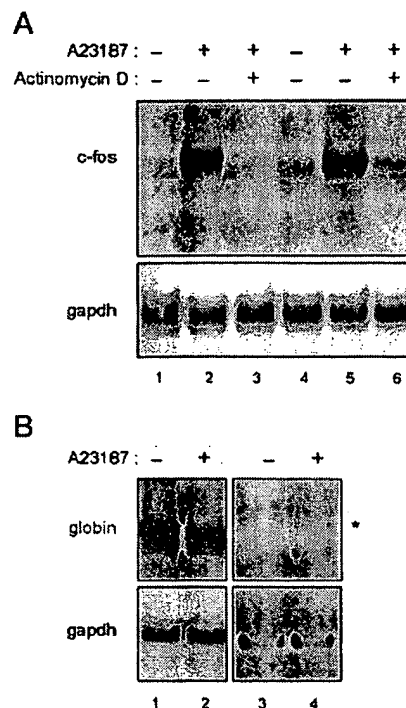


FIG. 4. *c-fos* intragenic sequences confer calcium inducibility to a heterologous promoter. *A*, exponentially growing Ltk⁻ cells, stably transfected with p19/1 (lanes 1–3) or p19/ΔNsiI-MstII (lanes 4–6), were stimulated for 1 h with A23187, followed by actinomycin D as indicated. *B*, exponentially growing Ltk⁻ cells, stably transfected with either pMT-globin (lanes 1 and 2) or pFos-globin (lanes 3 and 4), were induced for 1 h with A23187 as indicated. RNA from cells left untreated (-) or treated as indicated (+) was processed for Northern blot analysis as described in the legend of Fig. 1. A mouse *c-fos* cDNA probe was used in *A* and a rabbit genomic DNA probe was used in *B*. In all cases membranes were rehybridized to a rat *gapdh* probe for normalization. The band marked by an asterisk in the upper panels of panel *B*, lanes 3 and 4, represent a globin splicing intermediate (53).

Ltk⁻ cells was completely abolished by preincubation with the elongation inhibitor DRB (data not shown).

In order to confirm the involvement of the transcriptional pause site in this effect, we generated a new series of deletions restricted to intron 1 (Fig. 7*C*). Stably transfected pools of Ltk⁻ cells were established as above and used for *in vitro* run-on experiments (Fig. 7*A*). Dot blot hybridizations were performed on DNA from each pool of cells in order to ensure that the copy number of each transfected construct (which ranged from 20 to 30) was high enough to render insignificant the signal arising from the endogenous gene. This was confirmed by run-on analysis (data not shown). Although not as pronounced as for the endogenous gene (Fig. 2), the transgene still harbored a block to transcriptional elongation at the level of basal transcription (Fig. 7). Mutant Δ 1 showed the same biased hybridization signal to fragment A observed with the wild type gene (Fig. 7, *A* and *B*), which is indicative of a transcriptional pause (see above). Notably the signal became less biased upon further deletion (compare white bars (fragment A) and black bars (fragment B) in Fig. 7*B*), suggesting the gradual alleviation of transcriptional pausing. This confirms the key role played by intron 1 sequences in *c-fos* regulation in Ltk⁻ cells.

***c-fos* Induction by Calcium in Ltk⁻ Cells Does Not Involve Calmodulin-activated Kinases II and IV or Calcineurin**—A major mechanism by which an increase in intracellular calcium concentration regulates cellular events is through its association with calmodulin (CaM). The calcium-CaM complex binds to and modulates the activity of multiple regulatory molecules,

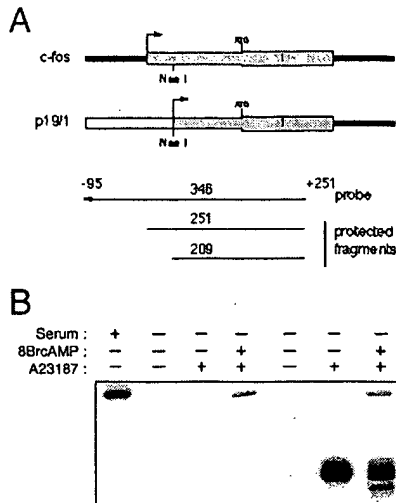


FIG. 5. RNase protection analysis of induction of the endogenous *c-fos* and exogenous MTLIIA-*fos* genes. **A**, schematic representation of the 5' portion of the endogenous *c-fos* gene as well as the chimeric gene encoded by p19/1. Shaded rectangles represent exons and black horizontal bars introns or flanking sequences. The open rectangle indicates the MTLIIA promoter in the chimeric construct. The horizontal arrow and the lines below positions the antisense RNA probe and indicates the expected protection products. These are bands of 251 and 209 nucleotides that correspond to transcripts generated from the endogenous gene and the transgenic construct, respectively. **B**, exponentially growing Ltk⁻ cells (lanes 1–4), or Ltk⁻ cells stably transfected with p19/1 (lanes 5–7), were stimulated for 1 h with A23187 alone (lane 3 and 6) or together with 8-Br-cAMP (lanes 4 and 7) as indicated. 20 μ g of RNA from uninduced (–) or induced (+) cells was hybridized and processed for RNase protection. Lane 1 shows the result using RNA from serum restimulated cells. The results shown are representative of three independent experiments. The faster migrating band of lane 7 is an artifact not observed in other analyses.

including the CaM-activated kinase family (CaM-K) (54). CaM-Ks appear to play a role in transcriptional activation because the calcium-dependent induction of several immediate early genes, such as *c-fos*, is blocked by the CaM-K inhibitors KN62 and KN93 (26, 55). This has been proposed to occur through CREB phosphorylation (44, 56, 57), SRF phosphorylation (28), and via an interaction between the CaM-K cascade and mitogen-activated protein kinase signaling pathways (58). We therefore investigated these possibilities in our experimental system.

Consistent with previous studies (59), treatment of p19/1 cells with calcium ionophore led to increased CREB phosphorylation, as measured by Western blotting and immunodetection with anti-phospho-CREB antibodies (data not shown). In contrast, A23187 did not affect the level of activated ERK1 and ERK2, as monitored on the same blot with antiphospho-ERK antibodies (not shown). Thus ionophore does not induce the ERK cascade in Ltk⁻ cells. Ionophore addition did stimulate CaM-K activity, as demonstrated with a pull-down kinase assay using calmodulin-affinity resin (Fig. 8A). A 1-h treatment of cells with KN93 not only abolished ionophore-mediated increase in CaM-K activity, but led also to a 70% decrease in basal activity (Fig. 8A). However, this did not inhibit *c-fos* mRNA induction in p19/1 cells (Fig. 8B).

This was somewhat surprising in light of previously published reports. To confirm the lack of CaM-K effect on *c-fos*, we transfected p19/1 cells with pHook-based vectors expressing constitutively active CaM-KII and CaM-KIV kinases. Transfected cells were enriched by immunoselection, and RNA and proteins processed for Northern and Western blot analysis. Consistent with the results above, neither CaM-KII nor CaM-KIV were able to recapitulate *c-fos* induction by calcium ionophore (Fig. 9A). In contrast, they were both able to increase phosphorylation of endogenous CREB (Fig. 9B). Furthermore, overexpression of inactive CaM-KII, which should compromise activation driven by CaM-KII, did not block *c-fos* induction by calcium ionophore (Fig. 9A) but did prevent A23187-induced CREB phosphorylation (Fig. 9B).

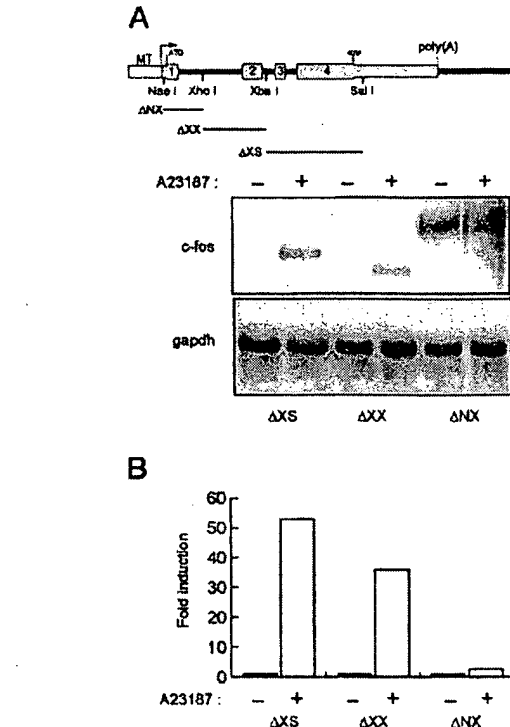


FIG. 6. Sequences contained within exon 1 and/or intron 1 of *c-fos* are required for a robust response to calcium. **A**, the upper panel diagrams the deletions introduced into the chimeric *c-fos* gene encoded by p19/1. The symbols are described in the legend to Fig. 5, and the bars under the gene represent the various deletions. Exponentially growing Ltk⁻ cells stably transfected with the three deletion mutants were left uninduced (–) or induced for 1 h with A23187 (+). Induction was analyzed by Northern blotting as described in the legend to Fig. 1. **B**, quantitative representation of induction. The hybridization signals in Panel A were analyzed densitometrically and normalized to the *gapdh* signal.

phore (Fig. 9A). In contrast, they were both able to increase phosphorylation of endogenous CREB (Fig. 9B). Furthermore, overexpression of inactive CaM-KII, which should compromise activation driven by CaM-KII, did not block *c-fos* induction by calcium ionophore (Fig. 9A) but did prevent A23187-induced CREB phosphorylation (Fig. 9B).

The calcium-CaM complex binds also to calcineurin, a calcium-dependent protein phosphatase implicated in gene regulation (38, 60). The immunosuppressant cyclosporin A is a potent calcineurin antagonist, whereas rapamycin, another immunosuppressant, acts independently of the phosphatase (60). We therefore checked whether cyclosporin A might influence A23187-mediated *c-fos* induction. Neither immunosuppressant had a notable effect on a wide range of concentrations (Fig. 10), therefore ruling out calcineurin in the effects we observe.

Calcium-mediated *c-fos* Induction Can be Mimicked by the Functional Inactivation of CaM—These data eliminated ERKs, CaM-Ks, and calcineurin as effectors of ionophore induction of *c-fos*. In order to evaluate the possible involvement of CaM in another pathway, we treated the cells with the anti-CaM drug W7 that has been extensively used to inhibit CaM in culture cell systems. As a control, we used W5, a drug chemically very similar to W7 but with a much lower affinity for CaM. To our surprise, exposure of cells to W7 prior to A23187 strongly induced *c-fos* either with or without A23187, while the control compound W5 was inactive (Fig. 11). This suggests that a novel calcium dependent pathway, antagonized by CaM, is involved in controlling transcriptional pausing in the murine *c-fos* locus.

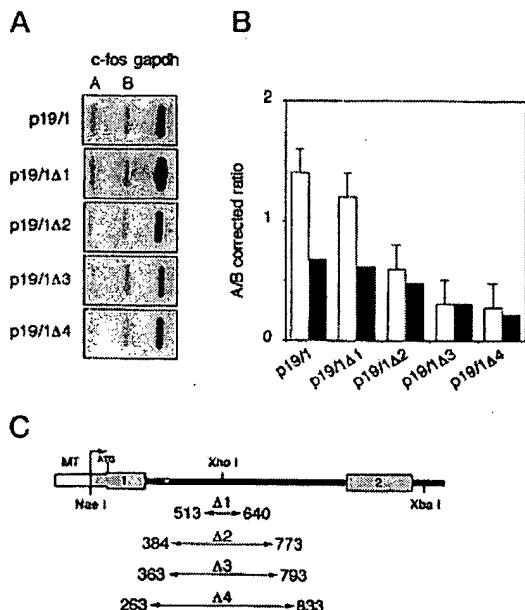


FIG. 7. The transcriptional pause site targeted by calcium ionophore maps in the 5' part of *c-fos* intron 1. *A*, nuclei were prepared from exponentially growing Ltk⁻ cells stably transfected with the various deletion mutants of p19/1. Run-ons were performed as indicated in Fig. 2 and nascent transcripts were hybridized to the *c-fos* NaeI-XhoI (fragment A) and XhoI-XbaI (fragment B) probes as well as to *gapdh* as described in the legend to Fig. 2. Experiments have been carried out three times and only a representative example is shown here. *B*, ratios of the signals detected on fragments A and B. The hybridization signals in panel A were quantitated and normalized to *gapdh* (open bars). The theoretical ratio, based on uridine content of transcripts arising from each deletion mutant, is presented as the solid bars. *C*, schematic representation of the deletions introduced into intron 1 of p19/1. The symbols are described in the legend to Fig. 6. The horizontal lines below portray the overlapping deletions centered on the XhoI site. Numbers refer to positions relative to the transcription initiation site. The white box present in intron 1 between positions +363 and +387 shows the previously mapped *in vitro* transcriptional pause site (39).

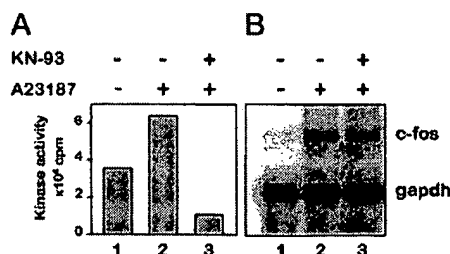


FIG. 8. CaM Kinase inhibitors do not affect *c-fos* mRNA induction by calcium ionophore. *A*, cells were pretreated with the CaM-K inhibitor KN93 (10 μ M, 10 min) and then stimulated with A23187 as indicated. Whole cell lysates were prepared and passed over a calmodulin affinity resin. The immobilized proteins were tested for kinase activity toward CaMtide, a CaM-K substrate peptide. Reactions were spotted on phosphocellulose paper, washed exhaustively, and the radioactivity measured by scintillation counting. *B*, cells were preincubated for 1 h with 10 μ M KN93, then induced as indicated above the lanes. Total RNA extraction, Northern blotting, and hybridization were performed as described in the legend to Fig. 1.

DISCUSSION

In this work, based on integrated *c-fos* reporter genes and not on transient transfection experiments, we show that calcium acts synergistically with other stimuli to activate *c-fos* transcription through both upstream and downstream regulatory elements. Whereas PMA, IBMX + cAMP, and A23187 alone were not sufficient to give rise to a significant activation of

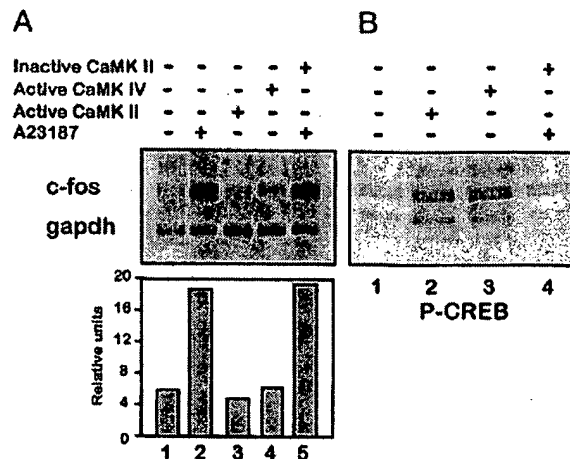


FIG. 9. Constitutively active CaM-Ks lead to CREB phosphorylation but fail to induce *c-fos* expression. Exponentially growing Ltk⁻ cells were transfected with either the parental vector (pHOOK-2-LacZ; lanes A1, A2, and B1) or expression vectors encoding constitutively active CaM-KII (CaM-KIIa-HOOK; lanes A3 and B2), constitutively active CaM-KIV (CaM-KIVa-HOOK; lanes A4 and B3), or inactive, dominant negative CaM-KII (CaM-KIII-HOOK; lanes A5 and B4). Transfected cells were selected on magnetic beads and lysed either with TRIzol for Northern blot analysis (panel A) or with SDS-polyacrylamide gel electrophoresis sample buffer for anti-phosphoCREB Western blotting (panel B, the membrane was stained with Ponceau Red to ensure identical loading). The lower panel presents the quantitation of the *c-fos* hybridization signals normalized to *gapdh*.

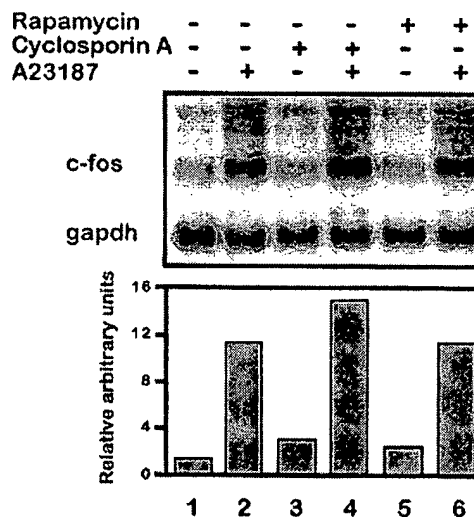


FIG. 10. Calcineurin does not contribute to calcium regulation of the *c-fos* intragenic pausing site. Exponentially growing Ltk⁻ p19/1 cells were pretreated with various concentrations of either cyclosporin A or rapamycin for 1 h (+) and then induced where indicated (+) with A23187. Only results obtained with 1 μ M of either drug are shown here. RNA isolation, Northern blotting, and hybridization were performed as described in the legend to Fig. 1. The lower panel shows the ratio of the *c-fos* signal to *gapdh* after PhosphorImager quantitation of the Northern blot.

c-fos, any combination of A23187 with the different inducers led to a strong induction of transcription. Notably upstream *c-fos* promoter sequences were not sufficient to confer a high level of inducibility by calcium to a reporter β -globin gene. Conversely, replacing the *c-fos* upstream promoter with that from the human metallothionein IIa promoter generated a chimeric gene highly inducible by calcium ionophore alone. Nuclear run-on analysis showed that mRNA accumulation was mainly due to a dramatic increase in polymerase processivity.

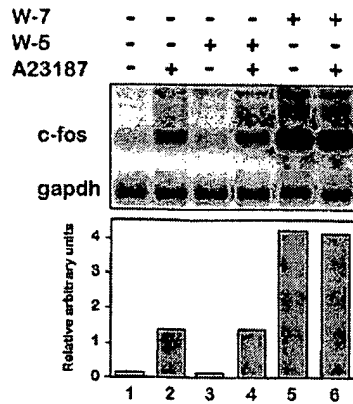


FIG. 11. A calmodulin antagonist disrupts regulation of the *c-fos* intragenic pausing site. Exponentially growing Ltk⁻ p19/1 cells were preincubated for 1 h with various concentrations of W5 or W7, followed by induction with A23187. Only results obtained with 25 μ M of either drug are shown here. RNA isolation, Northern blotting, and hybridization were performed as described in the legend to Fig. 1. The lower panel presents the quantitation of the *c-fos* hybridization signals normalized to *gapdh*.

These data suggest that intragenic regulatory elements mediate calcium effects provided that transcription initiation has taken place.

The pathway leading from calcium entry to transcription resumption was then evaluated: whereas CaM-Ks activate *c-fos* gene transcription through promoter proximal elements, astonishingly, their participation, as well as that of the calcium-dependent phosphatase calcineurin, was ruled out in the relief of the block to elongation. However, calmodulin is actually implicated in this phenomenon through a mechanism that remains to be characterized. Taken together, these arguments form a compelling body of data pointing to a new calcium/calmodulin-dependent pathway that targets intragenic sequences to allow transcription to proceed through the pause site.

We show here that the metallothionein promoter drives barely detectable transcription through *c-fos* downstream sequences. The striking induction of transcription upon A23187 treatment therefore reflects a tremendous increase in transcription elongation efficiency. Importantly the same result was obtained with other constitutively active promoters, such as the adenovirus major late or rat β -actin promoters (data not shown). This is consistent with run-on experiments carried out on cells stably transfected with mutants deleting intron sequences of *c-fos*, which further stressed the important role played by the intragenic transcriptional pause site previously identified *in vivo* (40, 48, 61) and mapped *in vitro* (39). Thus, in living cells, the first exon plus the 5' part of the first intron were shown to be crucial for the elongation block, whose release is responsible for calcium induction of the p19/1 construct.

These results confirm and extend the previous observation by Collart *et al.* (34) in macrophages and by Lee and Gilman (35) in murine T cells. In particular, we show that this calcium-sensitive block to elongation in the 5' part of the *c-fos* locus can act independently of the promoter but requires the whole coding unit structure to be fully active. Moreover, this phenomenon is no longer restricted to macrophages and T-cells but also applies in fibroblasts, suggesting that it reflects a mechanism with wider relevance than previously appreciated.

Interestingly, transcriptional induction was amplified by stabilization of *c-fos* mRNA when the endogenous gene was activated by a combination of A23187 and PMA or cAMP. *c-fos* transcripts generated by the various p19/1-derived constructs after stimulation by A23187 alone were stabilized to the same extent (data not shown), suggesting that calcium also modu-

lates *c-fos* mRNA decay directed by sequences in the 3'-untranslated region. This region cannot function alone, as shown by the inactive deletion mutants where it was still present. Furthermore, the fact that no deletion mutant retained full calcium-induced activity is consistent with data from transgenic mice showing that full *c-fos* inducibility *in vivo* requires the entire locus (62).

We then questioned which pathway led to elongation block release in response to calcium ionophore. Increased intracellular calcium can lead to activation of PKA and PKC, two important effectors of *c-fos* activation in culture cells and *in vivo*. The fact that ionophore could synergize with forskolin and PMA, strong activators of PKA and PKC, makes it unlikely that these two kinases mediate the effects we observe. Thus CaM-dependent kinases seemed the most likely candidates, since they have been implicated in mediating *c-fos* induction by several different signals and mechanisms (25, 26, 55, 63, 64). Surprisingly, the CaM-K inhibitor KN93 (nor KN-62: data not shown) did not affect *fos* induction although it inhibited both basal and calcium-induced CaM-K activity. Similarly overexpression of dominant-negative CaM-K did not block ionophore-driven p19/1 expression, which was also not reproduced by transfection of constitutively active CaM-Ks II or IV. In contrast, the latter did lead to CREB phosphorylation. This renders any role for CaM-Ks in this process very unlikely and suggests that their previously described activation of *c-fos* transcription takes place at the level of initiation rather than elongation.

The calcium-activated phosphatase calcineurin regulates gene expression by activating the nuclear localization of the cytoplasmic transcription factor NF-ATc (38, 60). This may account for certain signaling events attributed to cytoplasmic but not nuclear calcium fluxes (30). Nevertheless calcineurin does not mediate increased elongation driven by A23187, since the latter was insensitive to the calcineurin inhibitor cyclosporin A (65).

Recently, DREAM, a new repressor acting through a location-dependent silencer (DRE) has been shown to lead to a calcium-dependent repression of a human *c-fos* reporter in transient transfection experiments (32). The same sequence is present in the mouse locus but is not present in our reporter constructs that show calcium-dependent regulation. Therefore, the phenomenon we describe here is distinct from DREAM-dependent repression.

These experiments ruled out a number of well documented pathways activated by calcium. We thus tested whether the ionophore signal involved calmodulin itself, using the calmodulin antagonist W7. This compound led to a dramatic induction of the *c-fos* p19/1 transgene both with and without A23187. Thus this calmodulin antagonist acts in the same direction as calcium entry, which seems paradoxical at first sight. One possible explanation is that this reflects a calcium- and antagonist-sensitive interaction between calcium-free calmodulin and a factor responsible for the block to elongation. This mechanism might resemble that described previously for neuro-modulin (66, 67), a neurospecific protein whose function is believed to be to bind calcium-free calmodulin and concentrate it within specific regions of the neuron.

The nature of this downstream element and its regulation by calcium remain to be characterized. It might represent a downstream component of the promoter, inactive on its own, that interacts with a *bona fide* transcription factor. For example, some bHLH proteins have been shown to interact directly with and be regulated by calcium/calmodulin in their ability to bind DNA. Accordingly, calcium ionophore was shown to selectively inhibit transcriptional activation by these CaM-sensitive bHLH protein *in vivo* (68). Alternatively this phenomenon

might reflect a constrained chromatin structure responsive to calcium. This would explain why this effect has proven refractory to analysis by transient transfection. Given the quick response to calcium, one can imagine a direct interaction between calmodulin and an elongation factor, such as TFIIS, P-TEFb (cycI/cdk9), or elongin A, a chromatin remodeling system, *e.g.* Swi/Snf complex, or a histone acetyltransferase. Although the exact molecular mechanisms remain unclear, our data add a new, calcium-dependent pathway and promoter region to the complex signaling network that ensures the tight regulation of *c-fos* transcription.

Acknowledgments—We thank A. Means and S. Soderling for CaM-KII and -IV expression plasmids.

REFERENCES

- Almendral, J. M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) *Mol. Cell. Biol.* 8, 2140–2148
- Bravo, R. (1990) *Semin. Cancer Biol.* 1, 37–46
- Lau, L. F., and Nathans, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1182–1186
- Cahill, M. A., Janknecht, R., and Nordheim, A. (1996) *Curr. Biol.* 6, 16–19
- Janknecht, R. (1995) *Immunobiology* 193, 137–142
- Piechaczyk, M., and Blanchard, J. M. (1994) *Crit. Rev. Oncol. Hematol.* 17, 93–131
- Treisman, R. (1995) *EMBO J.* 14, 4905–4913
- Fisch, T. M., Prywes, R., and Roeder, R. G. (1987) *Mol. Cell. Biol.* 7, 3490–3502
- Gilman, M. Z., Wilson, R. N., and Weinberg, R. A. (1986) *Mol. Cell. Biol.* 6, 4305–4316
- Lucibello, F. C., Ehler, F., and Muller, R. (1991) *Nucleic Acids Res.* 19, 3583–3591
- Visvader, J., Sassone-Corsi, P., and Verma, I. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 9474–9478
- Hayes, T. E., Kitchen, A. M., and Cochran, B. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1272–1276
- Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. (1990) *EMBO J.* 9, 4477–4484
- Ginty, D. D., Bonni, A., and Greenberg, M. E. (1994) *Cell* 77, 713–725
- Greenberg, M. E., Siegfried, Z., and Ziff, E. B. (1987) *Mol. Cell. Biol.* 7, 1217–1225
- Velich, A., and Ziff, E. B. (1990) *Mol. Cell. Biol.* 10, 6273–6282
- Berkowitz, L. A., Riabowol, K. T., and Gilman, M. Z. (1989) *Mol. Cell. Biol.* 9, 4272–4281
- Fisch, T. M., Prywes, R., and Roeder, R. G. (1989) *Mol. Cell. Biol.* 9, 1327–1331
- Fisch, T. M., Prywes, R., Simon, M. C., and Roeder, R. G. (1989) *Genes Dev.* 3, 198–211
- Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L., and Verma, I. M. (1988) *Genes Dev.* 2, 1529–1538
- Sheng, M., Dougan, S. T., McFadden, G., and Greenberg, M. E. (1988) *Mol. Cell. Biol.* 8, 2787–2796
- Sheng, M., and Greenberg, M. E. (1990) *Neuron* 4, 477–485
- Bading, H., Ginty, D. D., and Greenberg, M. E. (1993) *Science* 260, 181–186
- Hipskind, R. A., Rao, V. N., Mueller, G. G., Reddy, E. S., and Nordheim, A. (1991) *Nature* 354, 531–534
- Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995) *Mol. Cell. Biol.* 15, 3672–3684
- Wang, Y., and Simonson, M. S. (1996) *Mol. Cell. Biol.* 16, 5915–5923
- Greenberg, M. E., Thompson, M. A., and Sheng, M. (1992) *J. Physiol. (Paris)* 86, 99–108
- Ginty, D. D. (1997) *Neuron* 18, 183–186
- Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) *Science* 273, 959–963
- Hardingham, G. E., Chawla, S., Johnson, C. M., and Bading, H. (1997) *Nature* 385, 260–265
- Sheng, M., McFadden, G., and Greenberg, M. E. (1990) *Neuron* 4, 571–582
- Carrion, A. M., Link, W. A., Ledo, F., Mellstrom, B., and Naranjo, J. R. (1999) *Nature* 398, 80–84
- Thompson, M. A., Ginty, D. D., Bonni, A., and Greenberg, M. E. (1995) *J. Biol. Chem.* 270, 4224–4235
- Collart, M. A., Tourkine, N., Belin, D., Vassalli, P., Jeanteur, P., and Blanchard, J. M. (1991) *Mol. Cell. Biol.* 11, 2826–2831
- Lee, G., and Gilman, M. (1994) *Mol. Cell. Biol.* 14, 4579–4587
- Werlen, G., Belin, D., Conne, B., Roche, E., Lew, D. P., and Prentki, M. (1993) *J. Biol. Chem.* 268, 16596–16601
- Collart, M. A., Belin, D., Briottet, C., Thorens, B., Vassalli, J. D., and Vassalli, P. (1989) *Oncogene* 4, 237–241
- Crabtree, G. R., and Clipstone, N. A. (1994) *Annu. Rev. Biochem.* 63, 1045–1083
- Mechti, N., Piechaczyk, M., Blanchard, J. M., Jeanteur, P., and Lebleu, B. (1991) *Mol. Cell. Biol.* 11, 2832–2841
- Plet, A., Eick, D., and Blanchard, J. M. (1995) *Oncogene* 10, 319–328
- Ruther, U., Wagner, E. F., and Muller, R. (1985) *EMBO J.* 4, 1775–1781
- Rech, J., and Fort, P. (1989) *Nucleic Acids Res.* 17, 2874
- Cruzalegui, F. H., Kapiloff, M. S., Morfin, J. P., Kemp, B. E., Rosenfeld, M. G., and Means, A. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 12127–12131
- Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) *Genes Dev.* 8, 2527–2539
- Hipskind, R. A., Baccarini, M., and Nordheim, A. (1994) *Mol. Cell. Biol.* 14, 6219–6231
- Li, S. L., Godson, C., Roche, E., Zhao, S. J., Prentki, M., and Schlegel, W. (1994) *J. Mol. Endocrinol.* 13, 303–312
- Ran, W., Dean, M., Levine, R. A., Henkle, C., and Campisi, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 8216–8220
- Sobczak, J., Mechti, N., Tournier, M. F., Blanchard, J. M., and Duguet, M. (1989) *Oncogene* 4, 1503–1508
- Fort, P., Rech, J., Vie, A., Piechaczyk, M., Bonni, A., Jeanteur, P., and Blanchard, J. M. (1987) *Nucleic Acids Res.* 15, 5657–5667
- Rahmsdorf, H. J., Schonthal, A., Angel, P., Litfin, M., Ruther, U., and Herrlich, P. (1987) *Nucleic Acids Res.* 15, 1643–1659
- Shyu, A. B., Greenberg, M. E., and Belasco, J. G. (1989) *Genes Dev.* 3, 60–72
- Chen, C. Y., Chen, T. M., and Shyu, A. B. (1994) *Mol. Cell. Biol.* 14, 416–426
- Bonni, A., Rech, J., Jeanteur, P., and Fort, P. (1989) *Oncogene* 4, 881–888
- Braun, A. P., and Schulman, H. (1995) *Annu. Rev. Physiol.* 57, 417–445
- Enslen, H., and Soderling, T. R. (1994) *J. Biol. Chem.* 269, 20872–20877
- Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994) *J. Biol. Chem.* 269, 15520–15507
- Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol.* 14, 6107–6116
- Enslen, H., Tokumitsu, H., Stork, P. J., Davis, R. J., and Soderling, T. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10803–10808
- Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) *Science* 252, 1427–1430
- Clipstone, N. A., and Crabtree, G. R. (1993) *Ann. N. Y. Acad. Sci.* 696, 20–30
- Lamb, N. J., Fernandez, A., Tourkine, N., Jeanteur, P., and Blanchard, J. M. (1990) *Cell* 61, 485–496
- Robertson, L. M., Kerppola, T. K., Vendrell, M., Luk, D., Smeyne, R. J., Bocchiaro, C., Morgan, J. I., and Curran, T. (1995) *Neuron* 14, 241–252
- Krebs, J., and Honegger, P. (1996) *Biochim. Biophys. Acta* 1313, 217–222
- Antoine, M., Gaidon, C., and Loeffler, J. P. (1996) *Mol. Cell. Endocrinol.* 120, 1–8
- Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997) *Genes Dev.* 11, 824–834
- Gamby, C., Waage, M. C., Allen, R. G., and Baizer, L. (1996) *J. Biol. Chem.* 271, 26698–26705
- Liu, Y. C., and Storm, D. R. (1990) *Trends Pharmacol. Sci.* 11, 107–111
- Corneliusson, B., Holm, M., Waltersson, Y., Onions, J., Hallberg, B., Thornell, A., and Grundstrom, T. (1994) *Nature* 368, 760–764

Retinal Capillary Pericyte Proliferation and c-Fos mRNA Induction by Prostaglandin D₂ through the cAMP Response Element

Shinichi Sakurai,¹ Shababuddin Alam,¹ Glorivee Pagan-Mercado,¹ Fatimah Hickman,¹ Jen-Yue Tsai,¹ Peggy Zelenka,² and Sanai Sato^{1,3}

PURPOSE. Cyclooxygenase inhibitors have been shown to prevent angiogenesis in some circumstances, suggesting that growth of capillary pericytes or endothelial cells may be regulated by prostaglandins (PGs). The present study tests the effects of PGs on the growth of human retinal capillary pericytes.

METHODS. Cell growth was assayed by formazan formation and 5-bromo-2'-deoxyuridine (BrdU) incorporation. The expression of mRNAs corresponding to *c-fos*, PG receptors, and VEGF was examined by RT-PCR. Signal transduction was evaluated by immunoblot analysis using phosphospecific antibodies against mitogen-activated protein kinases (MAPKs) and cAMP response element-binding protein (CREB). Synthesis of cAMP was inhibited with the adenylyl cyclase inhibitor SQ22536. A reporter gene (luciferase) assay was conducted using the expression vector pSVOAΔ5' containing the 379-bp *c-fos* promoter with and without a mutation in cAMP response element (CRE).

RESULTS. PGD₂ treatment induced *c-fos* mRNA, stimulated pericyte growth, and increased expression of VEGF mRNA. PGE₂ and -F_{2α} had similar effects on *c-fos* induction and pericyte growth, whereas PGI₂ was ineffective. RT-PCR confirmed that mRNAs corresponding to the receptors for PGD₂, -E₂, -F_{2α}, and -I₂ were expressed in human retinal pericytes. Stimulation by PGD₂ led to phosphorylation of CREB, but had negligible effect on phosphorylation of p44/42 MAPK. The adenylyl cyclase inhibitor inhibited CREB activation and *c-fos* induction by PGD₂. In a reporter gene assay, *c-fos* induction occurred only with wild-type *c-fos* promoter. Mutation in CRE eliminated the response to PGD₂.

CONCLUSIONS. PGD₂ promotes the growth of retinal capillary pericytes by signaling through cAMP and CREB. The findings underscore the importance of PGs in the growth of human retinal capillary pericytes and raise the possibility that PGs may play a role in proliferative retinopathies. (*Invest Ophthalmol Vis Sci.* 2002;43:2774-2781)

Proliferative vitreoretinopathy (PVR) develops in various retinal disorders, including retinal vein occlusion, retinopathy of prematurity, and diabetic retinopathy. PVR is one of the

major causes of blindness in industrial countries including the United States, Japan, and European countries. The underlying mechanism of PVR is the formation of new vessels that grow into the vitreous. Although matrix metalloproteinase(s) may initiate angiogenesis by destroying the basement membrane,¹ the endothelial cell growth factors play the key role in the formation of new vessels by stimulating endothelial cell growth.² Angiogenesis is also closely associated with chronic inflammation.³ The inflammatory response induced by the leaks and repeated hemorrhages from new vessels also play an important role in the further development of clinically apparent proliferative tissues.^{4,5}

The correlation between inflammation and angiogenesis suggests that inflammatory mediators, such as cytokines and prostaglandins (PGs), may play a role in the development of new vessels. In particular, there is mounting evidence implicating PGs and their synthetases (the cyclooxygenases [COXs]) in this process. For example, E-type PGs enhance angiogenesis in rabbits⁶ and in chicken embryos.⁷ Conversely, specific and nonspecific COX inhibitors inhibit cancer xenograft-induced angiogenesis⁸ and can eventually suppress cancer growth by limiting the blood supply.⁹⁻¹¹ COX inhibitors also inhibit the angiogenesis produced by oncostatin, a potent cytokine that has been shown to induce COX-2 expression.¹² Hypoxia, the major cause of retinal neovascularization, also induces COX-2 in endothelial cells.¹³ Although the exact mechanism(s) is not well understood, PGs and COX appear to regulate new vessel growth by regulating the expression of cytokines and endothelial growth factors.^{8,14,15} In particular, the induction of COX-2 by hypoxia is crucial for the later expression of the endothelial growth factor, VEGF.¹⁶

Retinal capillaries consist of two types of cells: endothelial cells and pericytes. Pericytes express muscle-type actins¹⁷ and provide appropriate tonus to maintain the functional structure of the capillary. Although pericytes have been shown to synthesize VEGF¹⁸ and regulate endothelial cell growth,¹⁹⁻²¹ relatively little is known about their response to PGs and other inflammatory mediators that are implicated in angiogenesis. However, several studies have shown that PGs and their synthetases modulate the growth of smooth muscle cells, a closely related cell type.¹⁷ For example, in atherosclerosis, a condition associated with smooth muscle cell proliferation, both COX-2 expression and PG production are increased.^{22,23} Moreover, COX-2 is a key factor in the growth of smooth muscle cells induced by both TNF-α and angiotensin II.²⁴ These findings led us to investigate whether PGs might have important regulatory effects on retinal pericytes, as well. Herein, we report that PGD₂ induced the expression of the early response gene, *c-fos*, and stimulated the growth of human retinal capillary pericytes through activation of the cAMP response element binding protein (CREB). In addition, our data indicate that PGD₂ increased the expression of VEGF mRNA, a key growth factor in retinal neovascularization.

From the Laboratories of ¹Ocular Therapeutics and ²Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland; and the ³Department of Medicine, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma.

Submitted for publication August 6, 2001; revised February 25, 2002; accepted March 26, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Sanai Sato, Department of Medicine, University of Oklahoma Health Science Center, PO Box 26901, BSEB 331, Oklahoma City, OK 73190-3048; sanai-sato@ouhsc.edu.

MATERIALS AND METHODS

Chemicals and Materials

Unless otherwise stated, all reagents used in the study were of analytical grade. PGD₂, -E₂, -F_{2α}, and -I₂, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Dulbecco's modified minimum essential medium (DMEM), fetal calf serum (FCS), trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in HEPES-buffered saline solution) and all other cell culture reagents were obtained from Biofluids, Inc. (Rockville, MD). Antibodies against phospho- and non-phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204) and phospho-p38 MAPK (Thr180/Tyr182) were the products of New England Biolabs, Inc. (Beverly, MA). Antibodies against non-phospho- and phospho-CREB (Ser133) and adenylyl cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) were purchased from Calbiochem Corp. (San Diego, CA). Transfection reagent (FuGENE6) and a dual-luciferase reporter assay system were obtained from Roche Diagnostics Corp. (Indianapolis, IN) and Promega Corp. (Madison, WI), respectively. Two human lymphocytic cell lines, Raji and SKW6.4, were obtained from American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics-BioWhittaker (San Diego, CA).

Human retinal capillary pericytes (from a 55-year-old white man) were obtained from Clonetics-BioWhittaker. Pericytes were maintained in DMEM supplemented with 10% FCS in the collagen-coated culture plates in a 5% CO₂ atmosphere. The identity of the cells was confirmed by detection of muscle type α -actins.¹⁷

Viable Cell Assay

The number of viable cells was measured spectrophotometrically on 96-well plate with a kit (CellTiter 96 Assay; Promega), according to the manufacturer's instructions.

Briefly, the suspended human pericytes (100 μ L) were plated into 96-well plates (2.5 \times 10⁴ cells per well) and cultured in DMEM containing various stimulants. After the cells were cultured at 37°C for 3 days, 15 μ L of dye (tetrazolium) solution was added, and the incubation at 37°C was continued for another 4 hours. After the cells were solubilized, the brown color developed by tetrazolium metabolism was measured at 570 nm. To eliminate the background absorbance by cell debris, the cell number, expressed as the absorbance at 570 nm, was adjusted by the absorbance at 650 nm. In each experiment, cells, cultured without and with 10% FCS were included as the negative and positive controls, respectively. The cell viability was expressed as the ratio of number of cell to that of the control cells cultured in serum-free DMEM.

Quantitation of DNA synthesis

DNA synthesis was assayed by quantitating 5-bromo-2'-deoxyuridine (BrdU) incorporation into newly synthesized DNA in 96-well plate using a cell proliferation ELISA kit (Roche Diagnostics Corp.). The cells (2.5 \times 10⁴ cells per well) were cultured in DMEM containing various stimulants at 37°C for 3 days. After labeling with BrdU for 4 hours, BrdU incorporated into DNA was spectrophotometrically quantitated at 370 nm after the reaction with a peroxidase-conjugated mouse monoclonal antibody against BrdU and the following peroxidase reaction with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The background absorbance was adjusted by absorbance at 492 nm.

RNA Preparation

Human pericyte mRNA was isolated by extraction reagent (QIAshredder; Qiagen, Valencia, CA) and a kit (RNeasy Mini Kit; Qiagen). All experiments were conducted according to the manufacturer's instructions. All mRNA preparations used in the study were confirmed to contain no genomic DNA and to produce no PCR products without reverse transcription. When PCR using PCR beads (Ready-To-Go beads; 0.2 mL tubes/plate; Amersham Pharmacia Biotech Inc., Piscataway, NJ)

TABLE 1. Primers and Predicted Product Sizes

Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	
Forward primer:	GTCAGTGGTGGACCTGACCT
Reverse primer:	AGGGGTCTACATGGCAACTG
Predicted product size:	420 bp
<i>c-Fos</i>	
Forward primer:	AGAATCCGAAGGGAAGGAA
Reverse primer:	CTTCTCTTACGACAGTTGG
Predicted product size:	150 bp
DP receptor	
Forward primer:	CTCTGCCCCGTAATTTATCGC
Reverse primer:	CACCGGCTCCTGTACCTAAG
Predicted product size:	214 bp
EP ₁ receptor	
Forward primer:	GTGTACATCCTACTGCGCCA
Reverse primer:	GGCTTTTATTTCCAAAGGC
Predicted product size:	247 bp
EP ₂ receptor	
Forward primer:	TGCTTCTCATTGTCTCGGTG
Reverse primer:	GTGAAAGGCAAGGAGCAGAC
Predicted product size:	229 bp
FP receptor	
Forward primer:	TTTGCCAATGGGAGGTAGAC
Reverse primer:	GGCAAAATAGGCCAGATCAA
Predicted product size:	263 bp
IP receptor	
Forward primer:	CTTCCAGCGACTCAAGCTCT
Reverse primer:	GAAATGTCAGCAGAGGGAGC
Predicted product size:	271 bp
Vascular endothelial growth factor (VEGF)	
Forward primer:	CTACCTCCACCATGCCAAGT
Reverse primer:	ATGTTGGACTCCTCAGTGGG
Predicted product size:	258 bp

produced any visible products, mRNA was treated with the DNase in a kit (DNA-free; Ambion, Austin, TX).

Reverse Transcription-Polymerase Chain Reaction

RT-PCR was conducted using RT-PCR beads (0.2 mL tubes/plate, Ready-To-Go; Amersham Pharmacia Biotech Inc.) on a PCR system (GeneAmp 9700; PE-Applied Biosystems, Foster City, CA). The primers and predicted molecular weights are summarized in Table 1.

Approximately 100 ng of mRNA was reverse transcribed and amplified in a total volume of 50 μ L with RT-PCR beads that contained 2.0 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 60 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, reverse transcriptase, porcine RNase inhibitor, and RNase- and DNase-free BSA. The condition for reverse transcription was 42°C for 30 minutes, and the amplification was performed in 32 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 30 seconds. The products were visualized by ethidium bromide after electrophoresis on 1% agarose gel.

Signal Transduction Assay

The cells were cultured in six-well culture plates (Costar Corp., Cambridge, MA) with regular DMEM containing 10% FCS. When the cells reached confluence, the medium was replaced with FCS-free DMEM. Culturing was continued for 12 hours and the cells were stimulated by incubating at 37°C for 10 minutes in DMEM containing appropriate stimulants. The cells were washed with ice-cold PBS and quickly frozen by placing the culture plate on dry ice. After the cells were solubilized with 1% SDS, the cell-signaling pathway was examined by detecting the phosphorylation of p44/42 MAPK, p38 MAPK, and CREB on Western blot analysis.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE was performed on a 12% acrylamide gel using a minigel system (XCell II electrophoresis apparatus; Novex, San Diego, CA).

After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) in Tris-glycine buffer (pH 8.3) containing 20% (vol/vol) methanol at 25 mV for 90 minutes with a blot transfer system (XCell II Blot Module; Novex). Nonspecific binding of the membrane was blocked with 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated in phosphate-buffered saline (PBS) containing 0.1% Tween-20, 5% BSA and primary antibody (rabbit IgG, 1:1000 dilution) at 25°C overnight and then in PBS containing 0.1% Tween-20, 5% nonfat dry milk, and secondary antibody (anti-goat IgG coupled with horseradish peroxidase, 1:1000 dilution) at 25°C for 3 hours. The immunostaining was detected by peroxidase reaction using a chemiluminescence detection system (Phototope-AP Western blot detection system; Cell Signaling Technology, Beverly, MA).

Reporter Gene Assay for Induction of *c-Fos* mRNA

Plasmids that carry the *c-fos* promoter coupled with the reporter gene luciferase and the same gene with mutation in the CRE were kindly provided by Bruce Cochran of Tufts University (Medford, MA) and Jianzhong Zhou of the National Eye Institute (Bethesda, MD). The wild-type *c-fos* promoter plasmid contains 379 bp of the *c-fos* regulatory region immediately 5' to the transcriptional start point, cloned upstream of the luciferase gene of the expression vector pSV0AΔ5'. In the mutant *c-fos* promoter plasmid, three nucleotides GTA (62-60) in the CRE region are mutated to TGG, as previously described.²⁵

The transfection into human retinal capillary pericytes was conducted in FCS-free DMEM for 8 hours, with transfection reagent (FUGENE6; Roche Diagnostic Corp.). After transfection, the cells were further incubated in serum-free DMEM for 24 hours and then in DMEM containing 1 μg/mL PGD₂ for another 24 hours. After the incubation, the cells were washed with PBS, quickly frozen by placing on dry ice, and stored at -20°C for luciferase assay. Luciferase activity was assayed with a dual-luciferase reporter assay system (Promega Corp., Madison, WI).

RESULTS

Stimulation of Pericyte Growth by PGs

To test whether PGs are able to stimulate pericyte growth, human retinal pericytes were cultured in serum-free medium containing two different concentrations (0.1 and 1 μg/mL) of PGD₂, -E₂, and -F_{2α} or in medium containing 10% FCS as a positive control. All three PGs significantly increased the number of cells in a dose-dependent manner (Fig. 1). Among these three PGs, PGD₂ was consistently the most effective, and the increase in cells with 1 μg/mL PGD₂ was approximately 60% as much as with 10% FCS. The increase in cells was statistically significant, even at 0.1 μg/mL. We also examined the effect of another PG, PGI₂, a well-known muscle dilator (Fig. 2). Unlike the other three PGs, PGI₂ caused a slight, but not statistically significant, decrease in the number of pericytes.

To confirm that the increase in cells by PGD₂ is due to increased cell growth rather than cell survival, DNA synthesis was also examined with a BrdU incorporation assay. When the cells were stimulated with 1 μg/mL PGD₂, DNA synthesis significantly increased (Fig. 3), confirming that the increase of cell number by PGD₂ is due to increased proliferation of pericytes.

Expression of PG Receptors

Because these results indicated that retinal pericytes responded to exogenous PGD₂ and two other PGs, PGE₂ and -F_{2α}, we next tested whether they express the necessary PG receptors (Fig. 4). RT-PCR detected mRNAs corresponding to the receptors for PGD₂ (DP), -E₂ (EP₁ and EP₂), -F₂ (FP), and -I₂ (IP), in human retinal capillary pericytes. The expression pat-

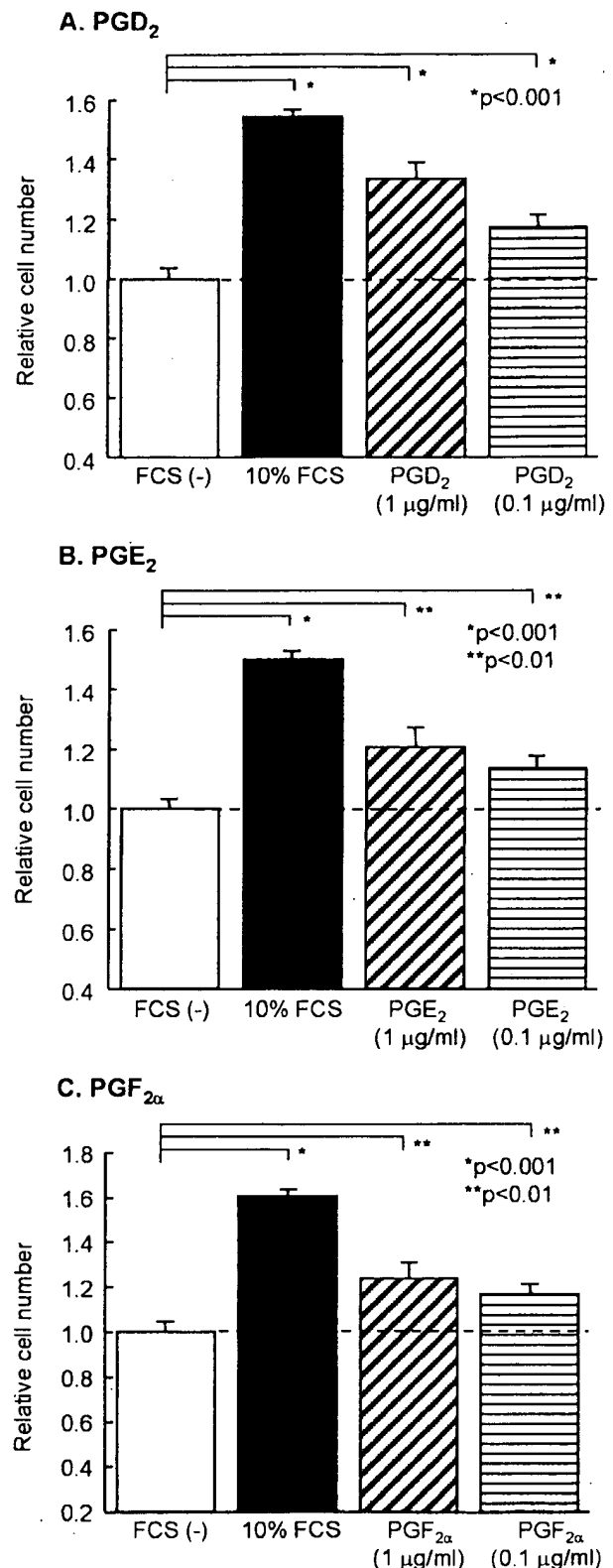


FIGURE 1. The effects of (A) PGD₂, (B) PGE₂, and (C) PGF_{2α} on the growth of human retinal capillary pericytes. Human pericytes were cultured in 96-well plates for 3 days in DMEM containing no growth factors (FCS(-)) or in media containing 10% FCS or 1 or 0.1 μg/mL PGD₂. The number of vital cells in each well was determined by the formation of formazan products. The data are expressed as the mean ratio of absorbance ± SEM at 570 nm against that of the control cells cultured without any growth factors (*n* = 6).

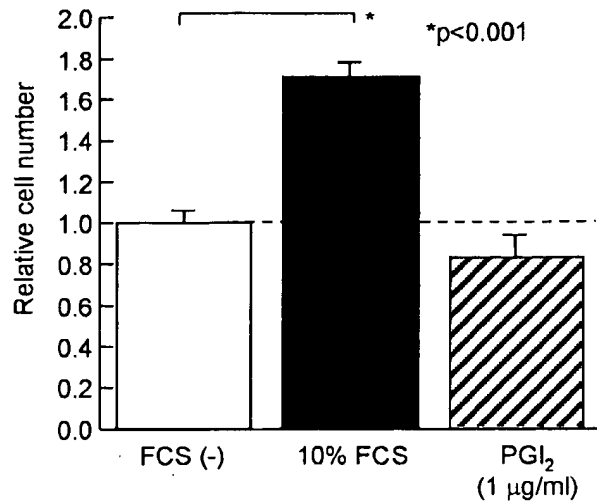


FIGURE 2. The effects of PGI₂ on the growth of human retinal capillary pericytes. Human pericytes were cultured in 96-well plates for 3 days in DMEM containing no growth factors (FCS(-)) or DMEM containing 10% FCS or 1 µg/ml PGI₂. The number of vital cells in each well was determined by the formation of formazan products. The data are expressed as the mean ratio of absorbance \pm SEM at 570 nm against that of the control cells cultured without any growth factors ($n = 6$).

tern of these receptors appeared to be cell-type specific. For example, DP and FP were not detected by RT-PCR in two lymphocytic cell lines, Raji and SKW6.4, although both were clearly present in human pericytes. In addition, the seven-transmembrane receptor CRTH2, which has been reported to mediate PGD₂-dependent cell migration of eosinophils and basophils,²⁶ was not detected by RT-PCR in human pericytes (data not shown).

Induction of the *c-Fos* Gene by PGD₂

To explore the mechanism by which PGs stimulate pericyte growth, we next examined the expression of the early-response gene, *c-fos*, by RT-PCR (Fig. 5). *c-Fos* mRNA was barely detected in human retinal capillary pericytes cultured for 12 hours in unsupplemented DMEM. However, when the cells

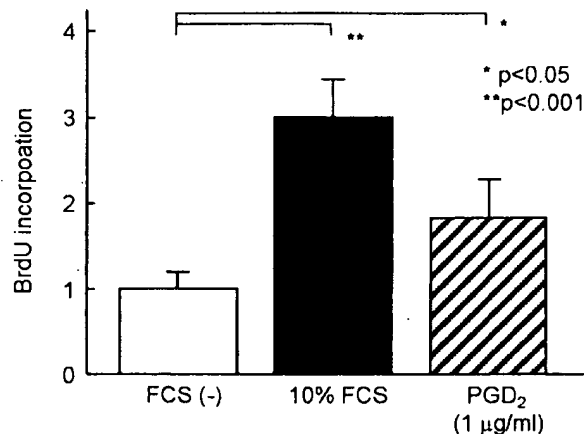


FIGURE 3. The effects of PGI₂ on DNA synthesis. Human pericytes were cultured in 96-well plates for 3 days in serum-free DMEM (FCS(-)) or DMEM containing 10% FCS or 1 µg/ml PGI₂. After labeling with BrdU for 4 hours, BrdU incorporated into newly formed DNAs was assayed spectrophotometrically at 370 nm. The data are expressed as the mean ratio of absorbance \pm SEM at 370 nm (adjusted by absorbance at 490 nm) against that of the control cells cultured in serum-free medium ($n = 6$).

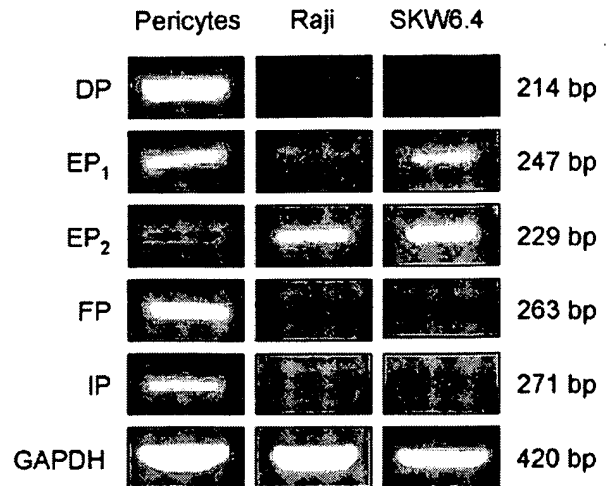
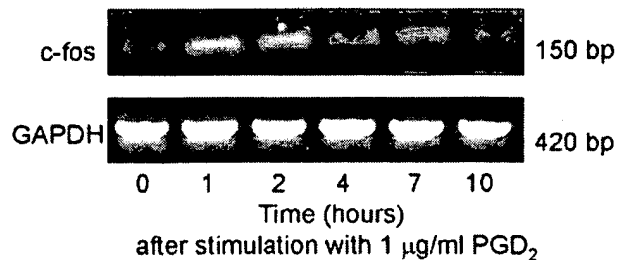


FIGURE 4. Expression of PG receptor mRNAs (RT-PCR) in human retinal capillary pericytes and two human leukemia cell lines, Raji and SKW6.4.

were stimulated by 1 µg/ml PGD₂, the expression of *c-fos* mRNA quickly increased, reaching a maximum at 1 hour after stimulation and then gradually decreasing (Fig. 5A). Both PGE₂ and -F_{2α} also strongly induced *c-fos* mRNAs (Fig. 5B), but PGD₂, which had the greatest effect on cell growth, also showed the strongest stimulation of *c-fos* mRNA. These findings suggest that stimulation of pericyte growth by PGs may be partly due to their ability to induce expression of the immediate early gene, *c-fos*.

A. Time course of *c-fos* mRNA expression by PGD₂



B. Induction of *c-fos* mRNA by FCS and PGs

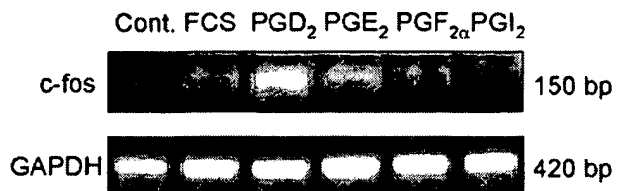


FIGURE 5. Expression of *c-fos* mRNA induced by FCS and various PGs (RT-PCR). (A) Time course of *c-fos* expression in human retinal pericytes after the stimulation by incubating in DMEM containing 1 µg/ml PGD₂ at 37°C. (B) *c-Fos* expression induced by stimulation with 10% FCS or 1 µg/ml PGD₂, -E₂, -F₂, or -I₂ at 37°C for 1 hour. Cont., no stimulant.

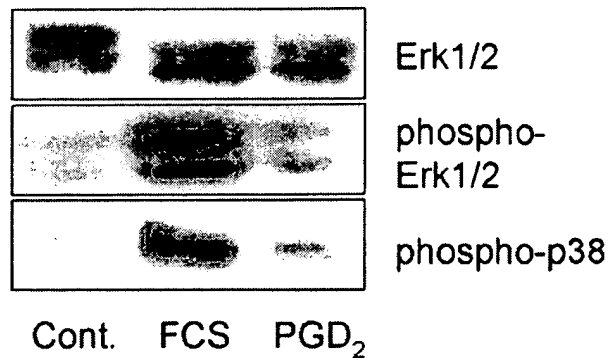


FIGURE 6. Activation of p44/42 MAPK (Erk1/2) and p38 MAPK by FCS and PGD₂. Human retinal capillary pericytes maintained in serum-free DMEM for 12 hours were stimulated by replacing the medium with DMEM containing no stimulant (Cont.), 10% FCS or 1 μ g/mL PGD₂ for 10 minutes. Activation of p44/42 MAPK and p38 MAPK was detected by Western blot analysis with antibodies against non-phospho- or phospho-p44/42 MAPK and -p38 MAPK.

Effects of PGD₂ on Activation of p44/42 MAPK (Erk1/2) and p38 MAPK

To explore the mechanism by which PGs induce *c-fos* mRNA and stimulate cell growth, we next examined the effect of PGD₂ on the phosphorylation state of the MAPKs, signaling enzymes involved in growth regulation in many cell types. Although PGD₂ strongly stimulated pericyte growth, we did not detect significant levels of the phosphorylated, active forms of p44/42 MAPK (Erk1/2) or p38 MAPK in cells treated with 1 μ g/mL PGD₂ (Fig. 6). In contrast, when human retinal capillary pericytes were stimulated by 10% FCS, the activation of both p44/42 and p38 MAPKs was clearly detected. Occasionally, slight positive staining with antibody against phospho-p44/42 MAPK was observed after stimulation by PGD₂. However, in view of the strong effect of PGD₂ on cell growth, it seemed unlikely that the MAPK pathway was the major signaling pathway used by this PG.

Activation of CREB by PGD₂

An alternative mechanism for induction of *c-fos* mRNA is activation of CREB. To test whether PGD₂ signaling might be mediated by CREB, we examined the relative concentration of the phosphorylated, active protein in the presence and absence of PGD₂ (Fig. 7). Phosphorylation of CREB was not detected in cells cultured in serum-free DMEM for 12 hours. However, when the cells were stimulated with 1 μ g/mL PGD₂, CREB was quickly activated. Phosphorylated CREB was detected within 5 minutes (Fig. 7A). Positive staining for phosphorylated CREB was continuously detectable for at least 1 hour and gradually decreased at later times. By 2 hours after the stimulation by PGD₂, the phosphorylated CREB was almost undetectable. When the adenylyl cyclase inhibitor SQ22536 was added to the medium, the activation of CREB by PGD₂ was significantly reduced (Fig. 7B).

Reduction of PGD₂-Induced *c-Fos* mRNA by Inhibition of Adenylyl Cyclase

To confirm the involvement of the cAMP signaling pathway in PGD₂-induced cell growth, we also investigated the effects of the adenylyl cyclase inhibitor SQ22536 on *c-fos* expression and cell growth induced by PGD₂. When pericytes were stimulated by 1 μ g/mL PGD₂, the induction of *c-fos* mRNA was clearly detected. This *c-fos* induction by PGD₂ was significantly reduced when SQ22536 was added to the medium (Fig. 8A). At a 1-mM concentration, SQ22536 almost completely eliminated

the induction of *c-fos* by PGD₂. SQ22536 also reduced FCS-dependent induction of *c-fos* mRNA (Fig. 8B), but this effect was minor when compared with the inhibition of PGD₂-dependent induction.

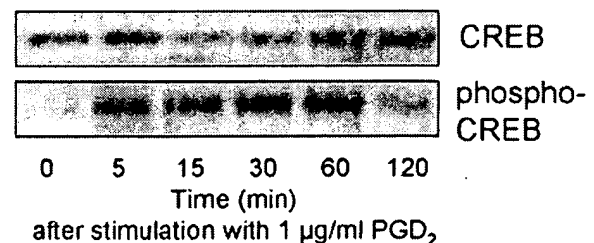
Reduction of PGD₂-Induced Cell Growth by Inhibition of Adenylyl Cyclase

In the absence of the adenylyl cyclase inhibitor SQ22536, the addition of 1 μ g/mL PGD₂ to the medium increased the number of human retinal pericytes by approximately 44%, compared with that cultured in serum-free and PGD₂-free medium for 3 days. When SQ22536 was added, PGD₂-induced cell growth was significantly reduced (Fig. 9). With 0.1- and 1-mM concentrations of SQ22536, the increase in cells by PGD₂ was only 20% and 16%, respectively. At both concentrations, the inhibition of PGD₂-induced cell growth by SQ22536 was statistically significant.

Elimination of PGD₂-Induced *c-Fos* Expression by Mutation in the CRE of the *c-Fos* Promoter

As an additional test of CREB involvement the induction of *c-fos* mRNA by PGD₂, the *c-fos* promoter gene, coupled with the luciferase reporter gene, was transfected into human retinal pericytes and *c-fos* mRNA induction by PGD₂ was examined (Fig. 10). No significant luciferase activity was detected in unstimulated cells transfected with either the wild-type *c-fos* promoter or the promoter containing a mutation in the CRE. However, when stimulated with 1 μ g/mL PGD₂, the cells carrying the wild-type *c-fos* promoter gene displayed significant luciferase activity, whereas those carrying the mutation in CRE did not (Fig. 10). Thus, an intact CRE site is required for the induction of *c-fos* by PGD₂.

A. Time course of CREB activation



B. Inhibition of CREB activation by SQ22536

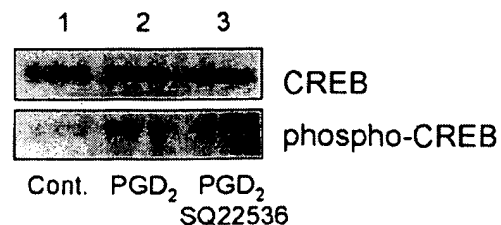


FIGURE 7. Activation of CREB by PGD₂. (A) Time course of CREB activation in human retinal capillary pericytes stimulated by 1 μ g/mL PGD₂ for 2 hours. (B) Inhibition of CREB activation by SQ22536. Human pericytes were stimulated by incubation for 10 minutes in serum-free DMEM (Cont.), DMEM containing 1 μ g/mL PGD₂, or medium containing 1 μ g/mL PGD₂ and 0.1 mM SQ22536. The activation of CREB was detected by Western blot analysis with antibodies against phospho- and non-phospho-CREB.

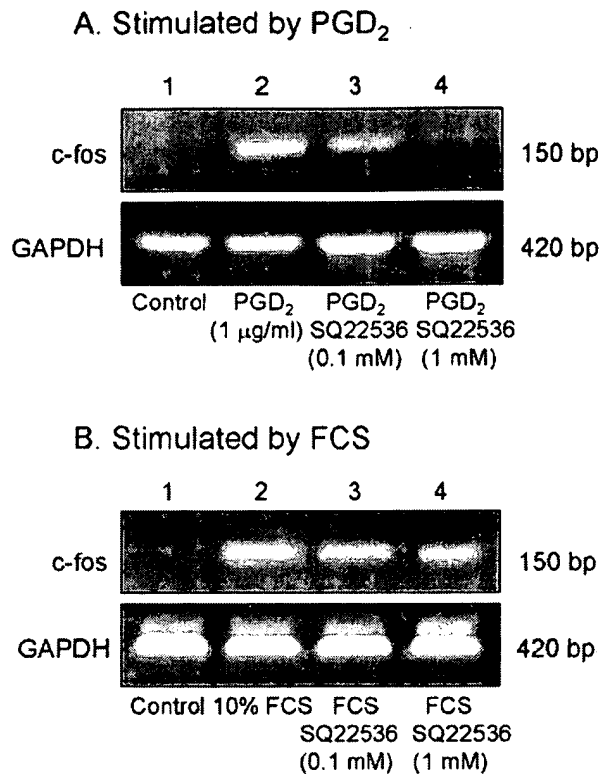


FIGURE 8. Inhibition of PDG₂-induced *c-fos* mRNA expression by adenylyl cyclase inhibitor SQ22536 (RT-PCR). (A) *c-fos* mRNA expression induced by PDG₂. Human pericytes maintained in FCS-free DMEM for 12 hours were stimulated by incubating at 37°C for 1 hour in serum free-DMEM (Control), the medium containing 1 µg/mL PDG₂, the medium containing both 1 µg/mL PDG₂ and 0.1 mM SQ22536, or the medium containing 1 µg/mL PDG₂ and 1 mM SQ22536. (B) *c-fos* expression by FCS. The same cells were incubated at 37°C for 1 hour in serum-free medium, medium containing 10% FCS, the medium containing 10% FCS and 0.1 mM SQ22536, or the medium containing 10% FCS and 1 mM SQ22536.

Induction of VEGF mRNA by PGD₂ in Human Retinal Pericytes

Because VEGF is a key growth factor for retinal neovascularization, we also investigated whether stimulation of pericyte proliferation by PGD₂ affects the expression levels of VEGF mRNA (Fig. 11). VEGF mRNA was barely detectable in resting pericytes. However, when human pericytes were stimulated with 1 µg/mL PGD₂, the relative concentration of VEGF mRNA steadily increased over a 7-hour period. In the presence of 1 mM SQ22536, this PGD₂ induction of VEGF mRNA was almost completely blocked (Fig. 12). This indicates that PGD₂ is a potent inducer of VEGF mRNA in retinal pericytes, and the cAMP pathway is also the major signaling pathway for the induction of VEGF mRNA.

DISCUSSION

PGD₂ is formed by isomerization of PGH₂, the primary product of arachidonic acid metabolism by cyclooxygenases. PGD₂ is further metabolized to 9α,11β-PGF_{2α} and/or the J series of prostanoids such as Δ¹²-PGJ₂ or 15-deoxy-Δ^{12,14}-PGJ₂ and displays a variety of physiological and pathologic functions, such as sleep induction, regulation of body temperature, hormone release, and nociception (see the recent review by Urade and Hayaishi²⁷). PGD₂ is especially important as the major mediator in allergic asthma.²⁸ It also has been reported that PGD₂ in-

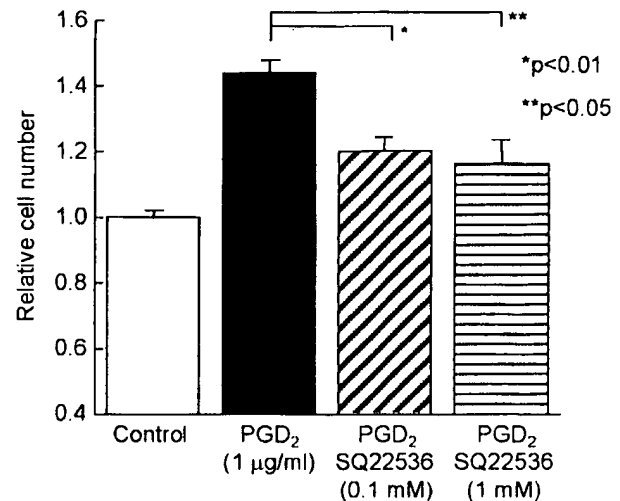


FIGURE 9. Inhibition of PDG₂-induced cell growth by adenylyl cyclase inhibitor SQ22536. Human retinal pericytes were plated into 96-well plates (2.5×10^4 cells per well) and cultured at 37°C for 3 days in 100 µL DMEM containing no growth factor (control), 1 µg/mL PDG₂, 1 µg/mL PDG₂, and 0.1 mM SQ22536, or 1 µg/mL PDG₂ and 1 mM SQ22536. The data are the relative mean number of cells \pm SEM, assayed by formazan metabolite formation, against the number of nonstimulated control cells ($n = 8$).

duces the early response genes *zif-268* and *tis-1* mRNAs in retinal pigment epithelial cells.²⁹ The present study also demonstrates that PGD₂ is essential as a stimulator of pericyte growth. Stimulation of PDG₂ induces rapid expression of *c-fos* mRNA and enhances the growth of retinal capillary pericytes. Although both PGE₂ and PGF_{2α} have displayed similar effects on pericyte growth, PGD₂ is more effective than those PGs in inducing the early response gene *c-fos* and in stimulating peri-

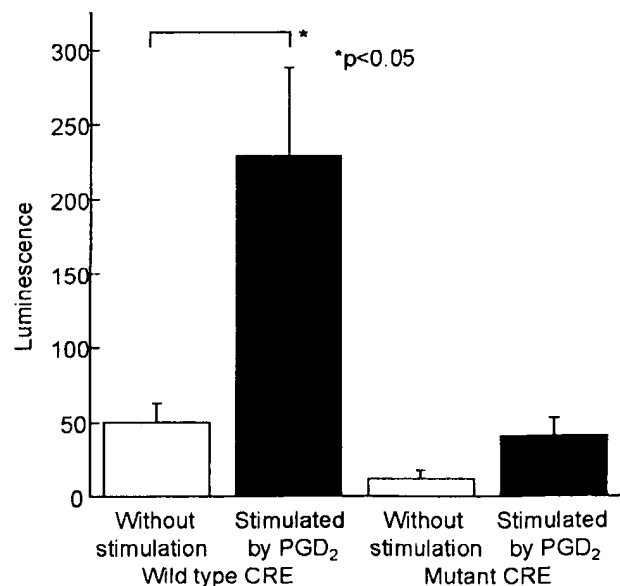


FIGURE 10. Reporter gene assay of *c-fos* expression by PGD₂. Human pericytes were transfected with the expression vector pSVOAΔ5' in which the *c-fos* promoter gene was inserted upstream of the luciferase gene. One of the plasmids contained the wild-type *c-fos* promoter (wild-type CRE), and the other contained the mutant CRE. After transfection, the cells were incubated in the presence or absence of 1 µg/mL PGD₂ for 24 hours, and mean luciferase activity \pm SEM was assayed using a dual-luciferase reporter assay ($n = 8$).

cyte growth. PGI₂, however, which is also formed from PGH₂, neither induced *c-fos* mRNA nor stimulated growth of human retinal pericytes. PGI₂ is structurally and functionally distinct from PGD₂, -E₂, and -F_{2 α} , and often opposes the action of these three PGs. For example, PGI₂ is a vessel dilator and inhibits the growth of smooth muscle cells, whereas PGE₂ and -F_{2 α} cause vessel contraction and stimulate the growth of smooth muscle cells.

Although PGD₂ and FCS both induce the expression of the early response gene *c-fos* and enhance the growth of capillary pericytes, the signaling pathways used appear to be distinct. It has been well established that p44/42 MAPK (ERK1/2) pathway is the major signaling pathway of cell growth and/or differentiation by various growth factors and lymphokines.^{30,31} Indeed, this study confirms that treating retinal pericytes with FCS activates p44/42 MAPK and, to a lesser extent, p38 MAPK. In contrast, the activation of p44/42 or p38 MAPK was barely detectable after stimulation by PGD₂, indicating that the MAPK pathway is unlikely to be the major signaling pathway in PGD₂-induced pericyte growth.

Our data indicate that the major signaling by PGD₂ in retinal pericytes goes through the cAMP pathway, which eventually activates the transcriptional regulator, CREB. PGs are known to bind to specific cell surface receptors, activating a G-protein cascade that leads to activation of adenylyl cyclase, increased levels of cAMP, and activation of protein kinase A (PKA).³² PKA, in turn, activates the transcription factor CREB,^{33,34} which has been implicated in *c-fos* induction in many cell types.^{35,36} The present study demonstrates that CREB is quickly activated when retinal pericytes are treated with PGD₂, and the phosphorylated CREB is detectable within 5 to 10 minutes. The adenylyl cyclase inhibitor SQ22536 significantly reduced PGD₂-dependent CREB phosphorylation and inhibited PGD₂-induced *c-fos* mRNA expression and cell growth. Moreover, in a reporter gene study, PGD₂ stimulated transcription of the wild-type *c-fos* promoter, but had no effect on transcription of the *c-fos* promoter with a mutation in the CRE site. To-

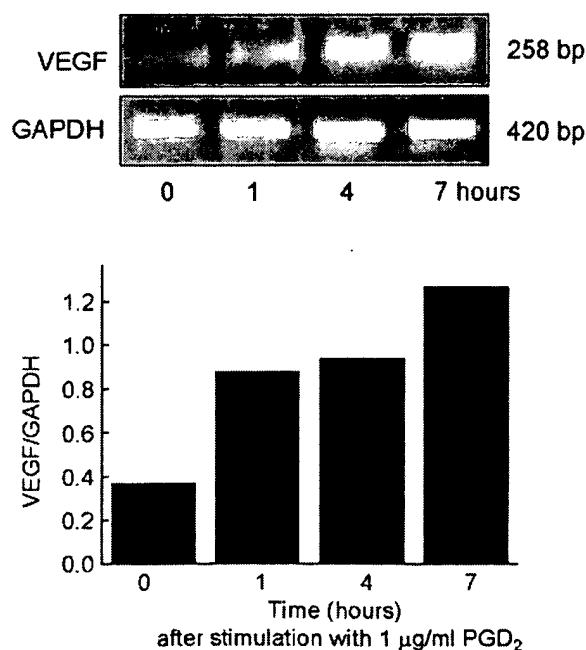


FIGURE 11. Induction of VEGF mRNA by PGD₂. Human pericytes maintained in serum-free medium for 12 hours were stimulated by replacing the medium with DMEM containing 1 µg/mL PGD₂. The VEGF mRNA was detected by RT-PCR. Data represent the ratio of the density of VEGF against that of GAPDH.

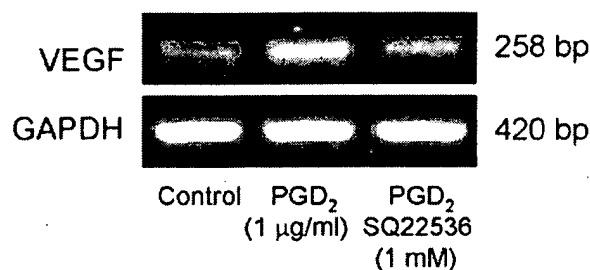


FIGURE 12. Inhibition of PGD₂-induced VEGF mRNA by the adenylyl cyclase inhibitor SQ22536 (RT-PCR). Human pericytes maintained in serum-free medium for 12 hours were stimulated for 5 hours by replacing the medium with DMEM containing 1 µg/mL PGD₂ in the presence or absence of 1 mM SQ22536.

gether, these findings support the view that the activation of CREB through the cAMP pathway is an essential early event in PGD₂-mediated *c-fos* induction and pericyte growth.

Although PGs are well-established inflammatory mediators, several observations suggest that PGD₂ also have important physiological roles in the eye. PGD₂ is widely detected in most eye tissues, including retina.³⁷ Although retinal pigment epithelium is the major site of PGD synthetase expression in rat retina, PGD synthetase activity is found at high levels in extracellular locations, such as interphotoreceptor matrix and vitreous and aqueous humors.^{38,39} The finding that DP receptor mRNA is expressed in epithelial cells of iris and ciliary body and in photoreceptor cells has led to the speculation that PGD₂ is important in regulation of intraocular pressure and in the vision process.⁴⁰ Considering the high levels of PGD₂ in retina, the finding that PGD₂ affects pericyte growth raises the possibility that it may be also important in maintaining the normal function of retinal capillaries.

Finally, in this study, PGD₂ treatment of retinal pericytes enhanced the expression of VEGF mRNA, a key growth factor in retinal neovascularization.² Recently, angiotensin II (Ang II), a factor known to activate CREB in rat smooth muscle cells,⁴¹ has been shown to induce VEGF expression in bovine retinal pericytes.¹⁸ Ang II-dependent VEGF induction can be eliminated by *c-fos* antisense,¹⁸ demonstrating that elevation of *c-fos* mRNA is necessary for VEGF induction. Indeed, many VEGF induction pathways lead to activation of the AP-1 transcription factor, of which c-Fos is a component.⁴² Together, these findings further support the view that CREB-dependent induction of *c-fos* mRNA is an important step in the induction of VEGF expression in retinal pericytes. Moreover, the finding that PGD₂ activates this CREB-dependent pathway in pericytes may provide a partial explanation for the known link between angiogenesis and chronic inflammation.³

References

1. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol.* 2001;21:1104-1117.
2. Aiello LP. Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. *Ophthalmic Res.* 1997;29:354-362.
3. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. *FASEB J.* 1997;11:457-465.
4. Tang S, Scheiffarth OF, Thureau SR, Wildner G. Cells of the immune system and their cytokines in epiretinal membranes and in the vitreous of patients with proliferative diabetic retinopathy. *Ophthalmic Res.* 1993;25:177-185.
5. Elner SG, Elner VM, Jaffe GJ, et al. Cytokines in proliferative diabetic retinopathy and proliferative vitreoretinopathy. *Curr Eye Res.* 1995;14:1045-1053.

6. Ziche M, Jones J, Gullino PM. Role of prostaglandin E1 and copper in angiogenesis. *J Natl Cancer Inst.* 1982;69:475-482.
7. Form DM, Auerbach R. PGE2 and angiogenesis. *Proc Soc Exp Biol Med.* 1983;172:214-218.
8. Sawaoka H, Tsuji S, Tsujii M, et al. Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth in vivo. *Lab Invest.* 1999;79:1469-1477.
9. Tsujii M, Kawano S, Tsuji S, et al. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell.* 1998;93:705-716.
10. Liu XH, Kirschenbaum A, Yao S, et al. Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer in vivo. *J Urol.* 2000;164:820-825.
11. Masferrer JL, Leahy KM, Koki AT, et al. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res.* 2000;60:1306-1311.
12. Pourtau J, Mirshahi F, Li H, et al. Cyclooxygenase-2 activity is necessary for the angiogenic properties of oncostatin M. *FEBS Lett.* 1999;459:453-457.
13. Schmedtje JF, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem.* 1997;272:601-608.
14. Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett.* 1995;372:83-87.
15. Ghosh AK, Hirasawa N, Niki H, Ohuchi K. Cyclooxygenase-2-mediated angiogenesis in carrageenin-induced granulation tissue in rats. *J Pharmacol Exp Ther.* 2000;295:802-809.
16. Liu XH, Kirschenbaum A, Yao S, et al. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clin Exp Metastasis.* 1999;17:687-694.
17. Herman IM, D'Amore PA. Microvascular pericytes contain muscle and nonmuscle actins. *J Cell Biol.* 1985;101:43-52.
18. Otani A, Takagi H, Oh H, et al. Angiotensin II-stimulated vascular endothelial growth factor expression in bovine retinal pericytes. *Invest Ophthalmol Vis Sci.* 2000;41:1192-1199.
19. Orlidge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol.* 1987;105:1455-1462.
20. Hirschi KK, D'Amore PA. Pericytes in the microvasculature. *Cardiovasc Res.* 1996;32:687-698.
21. Hirschi KK, D'Amore PA. Control of angiogenesis by the pericyte: molecular mechanisms and significance. *EXS.* 1997;79:419-428.
22. Schonbeck U, Sukhova GK, Graber P, Coulter S, Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. *Am J Pathol.* 1999;155:1281-1291.
23. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation.* 2000;102:840-845.
24. Young W, Mahboubi K, Haider A, Li I, Ferreri NR. Cyclooxygenase-2 is required for tumor necrosis factor-alpha- and angiotensin II-mediated proliferation of vascular smooth muscle cells. *Circ Res.* 2000;86:906-914.
25. Harvat BL, Wharton W. Serum response element and flanking sequences mediate the synergistic transcriptional activation of c-fos by 12-O-tetradecanoylphorbol-13-acetate and cholera toxin in AKR-2B cells. *Cell Growth Diff.* 1995;6:955-964.
26. Hirai H, Tanaka K, Yoshie O, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med.* 2001;193:255-261.
27. Urade Y, Hayaishi O. Prostaglandin D synthase: structure and function. *Vitam Horm.* 2000;58:89-120.
28. Matsuoka T, Hirata M, Tanaka H, et al. Prostaglandin D2 as a mediator of allergic asthma. *Science.* 2000;287:2013-2017.
29. Ershov AV, Parkins N, Lukiw WJ, Bazan NG. Modulation of early response gene expression by prostaglandins in cultured rat retinal pigment epithelium cells. *Curr Eye Res.* 2000;21:968-974.
30. Chao MV. Growth factor signaling: where is the specificity? *Cell.* 1992;68:995-997.
31. Adachi T, Nakashima S, Saji S, Nakamura T, Nozawa Y. Mitogen-activated protein kinase activation in hepatocyte growth factor-stimulated rat hepatocytes: involvement of protein tyrosine kinase and protein kinase C. *Hepatology.* 1996;23:1244-1253.
32. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev.* 1999;79:1193-1226.
33. Montminy M. Transcriptional regulation by cyclic AMP. *Annu Rev Biochem.* 1997;66:807-822.
34. Haus-Seuffert P, Meisterernst M. Mechanisms of transcriptional activation of cAMP-responsive element-binding protein CREB. *Mol Cell Biochem.* 2000;212:5-9.
35. Chen YQ, Gilliam DM, Rydzewski B, Naftilan AJ. Multiple enhancer elements mediate induction of c-fos in vascular smooth muscle cells. *Hypertension.* 1996;27:1224-1233.
36. Coulon V, Veyrune JL, Tourkine N, et al. A novel calcium signaling pathway targets the c-fos intragenic transcriptional pausing site. *J Biol Chem.* 1999;274:30439-30446.
37. Goh Y, Urade Y, Fujimoto N, Hayaishi O. Content and formation of prostaglandins and distribution of prostaglandin-related enzyme activities in the rat ocular system. *Biochim Biophys Acta.* 1987;921:302-311.
38. Beuckmann CT, Gordon WC, Kanaoka Y, et al. Lipocalin-type prostaglandin D synthase (beta-trace) is located in pigment epithelial cells of rat retina and accumulates within interphotoreceptor matrix. *J Neurosci.* 1996;16:9119-9124.
39. Gerashchenko DY, Beuckmann CT, Marcheselli VL, et al. Localization of lipocalin-type prostaglandin D synthase (beta-trace) in iris, ciliary body, and eye fluids. *Invest Ophthalmol Vis Sci.* 1998;39:198-203.
40. Gerashchenko D, Beuckmann CT, Kanaoka Y, et al. Dominant expression of rat prostanoid DP receptor mRNA in leptomeninges, inner segments of photoreceptor cells, iris epithelium, and ciliary processes. *J Neurochem.* 1998;71:937-945.
41. Funakoshi Y, Ichiki T, Ito K, Takeshita A. Induction of interleukin-6 expression by angiotensin II in rat vascular smooth muscle cells. *Hypertension.* 1999;34:118-125.
42. Kolch W, Martiny-Baron G, Kieser A, Marme D. Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. *Breast Cancer Res Treat.* 1995;36:139-155.

Angiotensin II-Stimulated Vascular Endothelial Growth Factor Expression in Bovine Retinal Pericytes

Atsushi Otani,¹ Hitoshi Takagi,¹ Hideyasu Oh,¹ Kiyoshi Suzuma,¹ Miyo Matsumura,¹ Eiji Ikeda,² and Yoshihito Honda¹

PURPOSE. Angiotensin II (AII) has been shown to play a role in many vascular diseases. In the study described, the effect of AII on vascular endothelial growth factor (VEGF) expression and related intracellular signaling mechanism was investigated in bovine retinal microcapillary pericytes.

METHODS. Cultured bovine retinal microvascular endothelial cells and pericytes were prepared. VEGF expression was determined by Northern blot analysis and immunoprecipitation assay. Cell proliferation was assessed by DNA content growth assay. Reporter gene studies were performed to identify the AII responsible transcription-activating region of VEGF gene.

RESULTS. Angiotensin II induced a significant increase in VEGF mRNA in a time- and dose-dependent manner. Angiotensin II type I receptor antagonist inhibited this effect. Angiotensin II activates the transcription of VEGF gene without changing the mRNA half-life, and the AII responsible region was found in the 5'-flanking region of the VEGF gene. Angiotensin II also increased the expression of c-fos and c-jun mRNA, and antisense oligonucleotides against c-Fos blocked the AII-induced VEGF mRNA expression. The conditioned media of AII-stimulated pericyte cultures had a growth-promoting effect on endothelial cells, and this effect was inhibited almost completely by VEGF neutralizing antibody.

CONCLUSIONS. These findings suggest that AII might induce angiogenic activity through a paracrine function of VEGF in retinal microvascular cells. (*Invest Ophthalmol Vis Sci.* 2000;41:1192-1199)

Retinopathy is a major complication of diabetes mellitus and is one of the leading causes of vision loss in developed countries.¹ Recent studies have shown that vascular endothelial growth factor (VEGF) plays a major role in the initiation and development of this particular form of retinopathy. VEGF is a potent angiogenic factor²⁻⁴ and vasopermeability factor⁵ and has been reported to generate a procoagulant state by induction of von Willebrand factor and a tissue factor.^{5,6} VEGF per se is sufficient to produce many of the vascular abnormalities common to diabetic retinopathy,⁷ and an increase in VEGF expression is seen in retinas of diabetic patients with little or no retinopathy.⁸ Furthermore, VEGF expression is increased by ischemia,⁹ and suppression of VEGF has been shown to inhibit neovascularization in animal models of retinal ischemia.^{10,11} VEGF levels are elevated also in patients with proliferative retinopathy and decrease after successful laser treatment,^{12,13} suggesting its importance in the early and proliferative stage of retinopathy.

Angiotensin II (AII) is known to be a key factor in cardiovascular homeostasis, and one that has many functions.¹⁴ An-

giotensin II also has a growth-promoting effect and has been reported to regulate the growth of vascular smooth muscle cells (SMCs)¹⁵ and to stimulate the induction of many growth factors.¹⁶⁻¹⁹ Based on these experimental data and clinical evidence, the renin-angiotensin system (RAS) is thought to play an important role in many cardiovascular disorders. Recent studies suggest that abnormalities in the RAS play a role also in the progression of diabetic nephropathy and retinopathy.²⁰⁻²⁴ In diabetic retinopathy, angiotensin-converting enzyme (ACE) inhibitors have been reported to improve the blood-retina barrier and to have favorable effects on patients with diabetic retinopathy.^{24,25} Furthermore, intraocular and serum levels of AII, prorenin, and ACE have been reported to be correlated with the severity of retinopathy.²⁰⁻²²

VEGF mediates its effects through endothelial cell-specific, high affinity phosphotyrosine kinase receptors: Flt-1 (VEGFR1)²⁵ and KDR/Flk-1 (VEGFR2).²⁵⁻²⁷ Recently VEGFs have been found to appear to interact with a neuronal cell-guidance receptor, neuropilin-1 (NP-1).²⁸ Previously, we reported that AII potentiates VEGF-mediated angiogenic activities of bovine retinal endothelial cells (BRECs) through upregulation of VEGFR2 expression, which suggests a substantial role for RAS in the pathogenesis of diabetic retinopathy.²⁹ In that study, we found no stimulatory effect of AII on VEGF expression in BRECs. In contrast, a stimulatory effect of AII on VEGF expression has recently been reported in human vascular SMCs³⁰ and rat heart endothelial cells.³¹

To further investigate how the RAS is involved in the pathogenesis of diabetic retinopathy, we determined the effect of AII on VEGF expression in bovine retinal microcapillary pericytes (BRPs), which are the other component cells of retinal microvasculature.

From the ¹Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan; and the ²Department of Pathology, Keio University School of Medicine, Tokyo, Japan.

Submitted for publication June 21, 1999; revised October 15, 1999; accepted November 16, 1999.

Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of the Japanese Government, and by the Japan Association for Inhibition of Blindness.

Commercial relationships policy: N.

Corresponding author: Hitoshi Takagi, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto 606-8397, Japan. hitoshi@kuhp.kyoto-u.ac.jp

METHODS

Cell Cultures

Primary cultures of BRPs and BRECs were isolated as previously described.³² Briefly, bovine retinas were homogenized and the homogenate was passed over 210-, 88-, and 53- μ m nylon meshes (Nippon Kikagaku Kikai, Tokyo, Japan). The materials caught up by the 53- and 88- μ m meshes were plated on the fibronectin-coated dishes (Iwaki, Tokyo, Japan). BRPs (fraction from 53- μ m mesh) were grown with Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS; Wheaton, Pipersville, PA). BRECs (fractions from both 88- and 53- μ m mesh) were cultured in DMEM with 5.5 mM glucose, 10% plasma-derived horse serum (PDHS; Wheaton), 50 mg/l heparin, and 50 U/l endothelial cell growth factor (Boehringer Mannheim, Indianapolis, IN). To keep homogeneity of the cells, contaminated cells were excluded by a weeding procedure.³³ When the cells reached subconfluence, BRPs were passaged after trypsinization, and cells from the 2nd and 3rd passages were used for the experiments after serum starvation with 0.5% PDHS for BRECs and 0.1% bovine serum albumin (BSA) for BRPs. For AII receptor antagonist studies, we used 1 μ M of AII type 1 receptor (AT₁)-specific antagonist Dup735 (Merck Research Laboratories, Rahway, NJ), a nonpeptide imidazole derivative³⁴ or nonpeptide AII type 2 receptor (AT₂) antagonist, PD123319 (Research Biochemicals International, Natick, MA)³⁵ for 15 minutes, followed by stimulation with AII for 3 hours. In all experiments we used vehicle (DMEM containing 0.1% BSA) as control.

Pericyte and Endothelial Cell Identification

Endothelial cell homogeneity was confirmed by immunoreactivity with anti-factor VIII antibodies (Dako, Glostrup, Denmark) analyzed by confocal microscopy. Pericyte homogeneity were confirmed by its characteristic features³³ and immunoreactivity with 3G5 monoclonal antibodies³⁶ (a generous gift from George L. King, Joslin Diabetes Center, Boston, MA) that was negative for SMCs. To avoid contamination of endothelial cells and glial cells, we confirmed the negative immunoreactivities for anti-factor VIII antibodies or anti-glial fibrillary acidic protein, respectively, by confocal microscopy.

Northern Blot Analysis

Total RNA was isolated from individual tissue culture plates using guanidine thiocyanate.³⁷ Northern blot analysis was performed on 15 μ g total RNA after 1% agarose-2 M formaldehyde gel electrophoresis and subsequent capillary transfer to Bio-dyne nylon membranes (Pall BioSupport, East Hills, NY) and ultraviolet cross-linking using a FUNA-UV-LINKER (model FS-1500; Funakoshi, Tokyo, Japan). Radioactive probes were generated using Amersham Megaprime labeling kits and ³²P-dCTP (DuPont, Wilmington, DE). Blots were prehybridized, hybridized, and washed in 0.5 \times SSC, 5% sodium dodecyl sulfate (SDS) at 65°C with 4 changes over 1 hour in a rotating hybridization oven (TAITEC, Koshigaya, Japan). All signals were analyzed using a densitometer (model BAS-2000II; Fuji Photograph Film, Tokyo, Japan), and lane loading differences were normalized using a 36B4 cDNA probe, which hybridizes to acidic ribosomal phosphoprotein PO.³⁸ Human VEGF cDNA (generously provided by Loyd P. Aiello, Boston, MA),³⁹ *c-fos* DNA (Takara shuzo, Shiga, Japan),⁴⁰ and *c-jun* cDNA (Calbiochem, La Jolla, CA)⁴¹ were used as probes.

Analysis of VEGF mRNA Half-Life

To determine whether the increase in VEGF mRNA was caused by an increase in transcription, BRPs were exposed to 5 μ g/ml actinomycin D (Wako, Osaka, Japan) after 3 hours of incubation with vehicle or AII (10 nM). The total RNA was then extracted, and Northern blot analysis was performed.

VEGF Protein Synthesis

Subconfluent cultures of BRPs were treated with 10 nM AII or vehicle for 3 hours. The culture media were then replaced with labeling media (DMEM minus methionine and cysteine, 100 μ Ci ³⁵S-methionine and cysteine) supplemented with AII or vehicle, as described above. After 2 hours' incubation, the medium was removed and the cells were lysed in solubilizing buffer (50 mM HEPES, pH 7.4, 10 mM EDTA, 100 mM NaF, 10 mM Na pyrophosphate, 1% Triton X-100, 10 mM NaVO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride) at 4°C for 1 hour. Protein concentrations were measured by the Pierce BCA procedure (BCA protein assay; Pierce, Rockford, IL). Specific antibody to VEGF (50 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the protein samples (500 μ g) and rocked at 4°C for 1.5 hours, and then 10 μ g protein A Sepharose was added and rocked for another 1.5 hours at 4°C. Protein A Sepharose antigen antibody conjugates were separated by centrifugation, washed 5 times, and boiled for 3 minutes in Laemmli sample buffer to denature. The samples were separated by 7.5% SDS-polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA), and the gel was vacuum dried. Results were visualized and quantified by a BAS-2000II densitometer (Fuji Photograph Film).

Reporter Gene Studies

A series of plasmid constructs were made from a genomic DNA clone of the human VEGF gene,⁴² which contained approximately 2.5 kb of the 5'-flanking region with the putative promoter and 1 kb of the 5'-untranslated region that was generously provided by Scios (Sunnyvale, CA).⁴³ These constructs have a series of deletion constructs of a region from 80 bp up to 3.2 kb upstream of the translation start site of the VEGF gene and subcloned upstream of the luciferase gene in the promoterless luciferase reporter vector pGL2-basic vector (Promega, Madison, WI), as shown Figure 3. As a control plasmid, we used renilla luciferase pRL-SV40 vector (Toyo Ink, Tokyo, Japan). Plasmids were transfected into BRPs by LipofectAMINE reagent (Life Technologies, Gaithersburg, MD). BRPs were seeded in 35-mm-diameter culture dishes (Iwaki) and incubated until the cells became subconfluent. A total of 1.5 μ g test plasmid and 0.05 μ g control plasmid was mixed with LipofectAMINE and added to the cells. After 5 hours' incubation, the mixture was replaced by normal growth medium and incubated an additional 20 hours. The cells were serum-deprived for 24 hours and then stimulated with 10 nM AII or vehicle for 18 hours. Cell extracts were then prepared by Lysis Buffer (Toyo Ink), and luciferase and renilla luciferase activity were measured by Luminoskan (Labsystems, Helsinki, Finland) with a Luciferase Dual Assay System (Toyo Ink). To standardize the transfection efficiency, luciferase activity was divided by renilla luciferase activity, and the degree of induction by AII for each test plasmid was determined as the ratio of standardized luciferase activity in AII-treated cells to that in vehicle-treated cells.

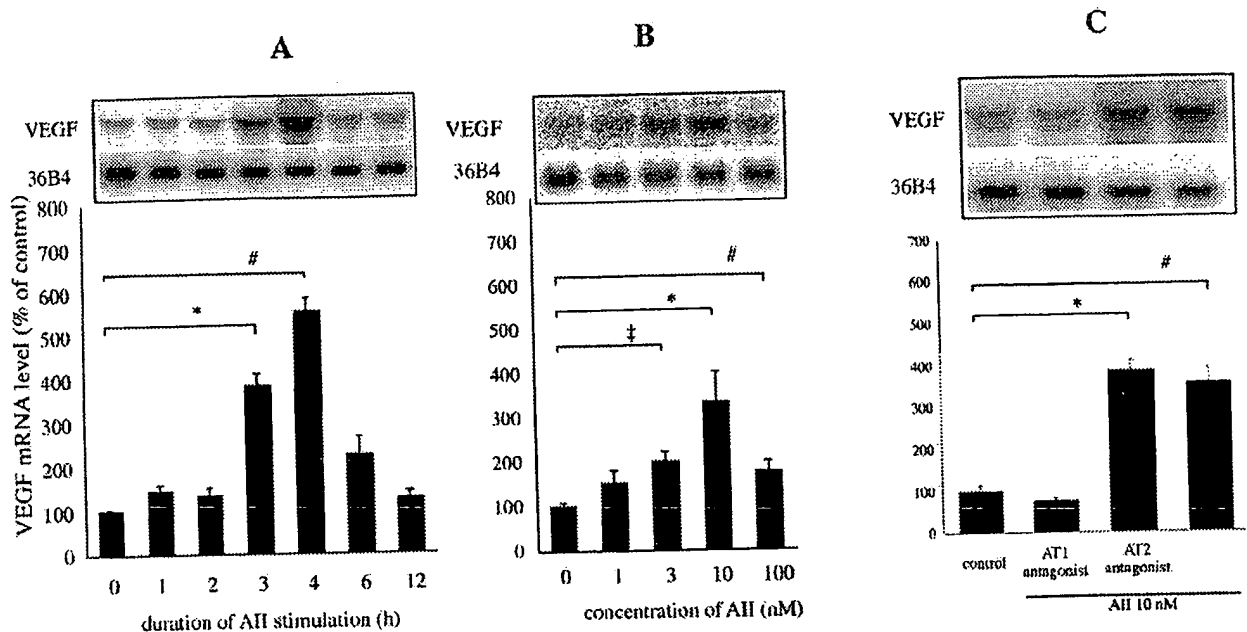


FIGURE 1. AII-stimulated VEGF mRNA expression in BRPs. Northern blot analysis (against 15 μ g/lane of total RNA) was performed with ³²P-dATP-labeled cDNA probes for VEGF. 36B4 probes were used to normalize the loading difference. The y-axis represents VEGF mRNA level expressed as percentage of control, and results are expressed as mean \pm SE. (A) Time course study ($n = 5$, $\#P < 0.01$, $*P < 0.01$), (B) dose-response study of 3-hour time point ($n = 3$, $\#P < 0.05$, $*P < 0.01$, and $\ddagger P < 0.01$), (C) effect of AT₁ and AT₂ antagonists on AII-stimulated (10 nM, 3 hours) VEGF mRNA expression ($n = 3$, $\#P < 0.05$, $*P < 0.05$). Representative blots from three experiments are shown (*top*).

Antisense Oligonucleotide Inhibition

Antisense oligonucleotides to the 5' ends of *c-fos* (5'-TGCGT-TGAAGCCCCGAGAA-3') and SP-1 (5'-CACCACAGCTGTCATTTCATCCATGG-3') and the corresponding sense oligonucleotides that were purified by high-performance liquid column chromatography were prepared. As described before,⁴⁴ these oligonucleotides were transfected into the cells without any treatment. After cells were incubated with DMEM containing 1% FBS for 40 hours, 5 μ M oligonucleotides were added and incubated an additional 8 hours. The cells were then washed with serum-free DMEM and incubated with or without 10 nM AII for 4 hours.

BREC Growth Assay

Serum-deprived BRPs were treated with AII (10 nM) or vehicle for 24 hours, and the conditioned medium was prepared. BRECs were plated in 24-well plates (Iwaki) at a density of 3×10^3 cells/well in DMEM containing 10% calf serum (GIBCO, Grand Island, NY). After 24 hours at 37°C, the medium was replaced with the conditioned medium. After 4 days' incubation,⁴⁵ the cells were lysed and DNA concentrations in each well were measured by DyNA Quant 200 (Hofer, San Francisco, CA).

Statistical Analysis

Determinations were performed in triplicate, and experiments were performed at least three times. Results were expressed as mean \pm SE, unless otherwise indicated. For multiple treatment groups, a factorial ANOVA followed by Fisher's least significant difference test was performed. Statistical significance was accepted at $P < 0.05$.

RESULTS

Effects of AII on VEGF mRNA Expression in BRPs

From results of several independent experiments it was clear that the effect of AII (10 nM) was time-dependent, with a maximal 6.5 ± 0.4 -fold increase at 4 hours ($P < 0.01$), which diminished progressively up to 12 hours (Fig. 1A). To define the concentration dependency of AII-induced VEGF mRNA expression in BRPs, we used the 3-hour time point. In this experiment, AII, 3 to 100 nM, significantly stimulated the induction of VEGF mRNA with an EC₅₀ of approximately 3 nM and a maximal 3.1 ± 0.7 -fold ($P < 0.01$) increase at 10 nM (Fig. 1B). AT₁ antagonist but not AT₂ antagonist inhibited the AII-induced VEGF mRNA expression ($P < 0.05$; Fig. 1C).

Effect of AII on Half-Life of VEGF mRNA

Cells were treated with actinomycin D (5 μ g/ml), a de novo gene transcription inhibitor, and Northern blot analysis was performed to measure VEGF mRNA levels. The half-life of VEGF mRNA without AII was 2.1 hours, and after AII exposure the half-life had not changed significantly (Fig. 2).

AII-Responsible Region in 5'-Flanking Region of VEGF Gene

Induced luciferase activity by AII was observed in cells transfected with *KpnI-NarI* (-3317 to -81), *SpeI-NheI* (-2848 to -984), and *SacI-NheI* (-2218 to -984) sites; however, no induction was observed in *BanI-NheI* (-1925 to -984), *PstI-NheI* (-1828 to -984), or *ApaI-NheI* (-1169 to -984) fragment-transfected cells (Fig. 3). Two plates were transfected for each test plasmid at the same time, and three independent experiments were performed. From these data it appears that

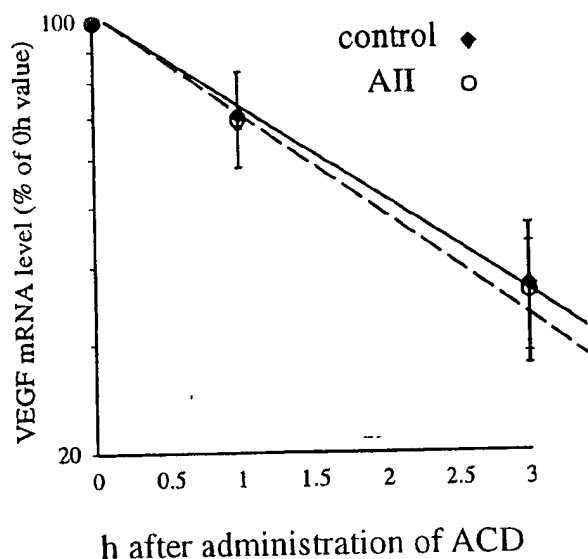


FIGURE 2. Effect of actinomycin D (ACD) on VEGF mRNA expression in response to AII in BRPs. BRPs were exposed to either vehicle or AII (10 nM) for 3 hours, and *de novo* mRNA transcription was inhibited by the addition of ACD 5 μ M. Total RNA was extracted at 1 hour and 3 hours, and Northern blot analysis was performed to detect VEGF mRNA level. The y-axis represents VEGF mRNA level and x-axis represents time after treatment. Each plot is a percentage of 0 hour value in logarithmic scale. The half-lives are indicated by drawing a line at the 50% point. Data from three independent experiments ($n = 3$) are shown.

an AII-responsive region is located in the *SacI*-*BanI* fragment (293 bp, -2218 to -1926, indicated by the solid underline in Fig. 3).

AII-Stimulated *c-fos* and *c-jun* mRNA Expression in Pericytes

The AII-responsive fragment of the VEGF gene that we found in this study contains potential binding sites for transcription

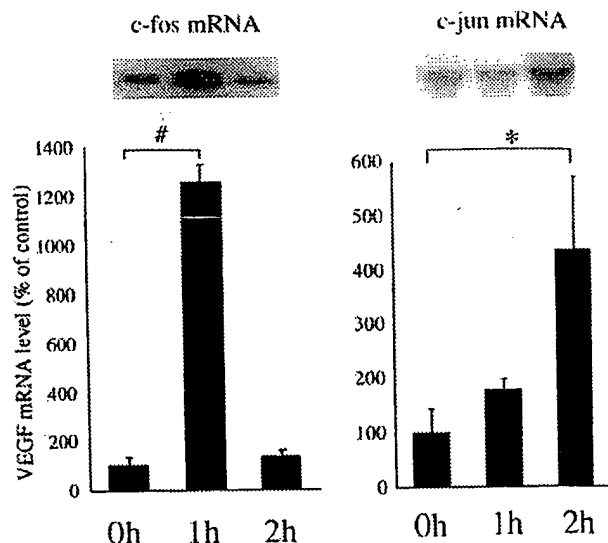


FIGURE 4. AII-stimulated *c-fos* and *c-jun* mRNA expression in BRPs. Total RNA was isolated at the indicated times after being stimulated with 10 nM AII. Northern blot analysis was performed with cDNA probes for *c-fos* and *c-jun*. Representative blots from three experiments ($n = 3$, $\#P < 0.0001$, $*P < 0.0001$) are shown (top).

factors SP-1, AP-1, and HIF-1.^{33,37} To investigate the role of AP-1 in AII-induced VEGF expression, we performed Northern blot analysis. By stimulation with 10 nM AII, 12.5 ± 0.7 -fold ($P < 0.001$, at 1 hour) and 4.4 ± 0.1 -fold ($P < 0.001$, at 2 hours) increases in *c-fos* and *c-jun* mRNA expression were observed, respectively (Fig. 4).

Inhibition of VEGF mRNA Production by Antisense Oligonucleotides Against c-Fos

To further examine the transcriptional factors involved in AII-induced VEGF expression, we assessed the effects of antisense oligonucleotides targeted against c-Fos and SP-1. BRPs were pretreated with 5 μ M oligonucleotides for 8 hours before

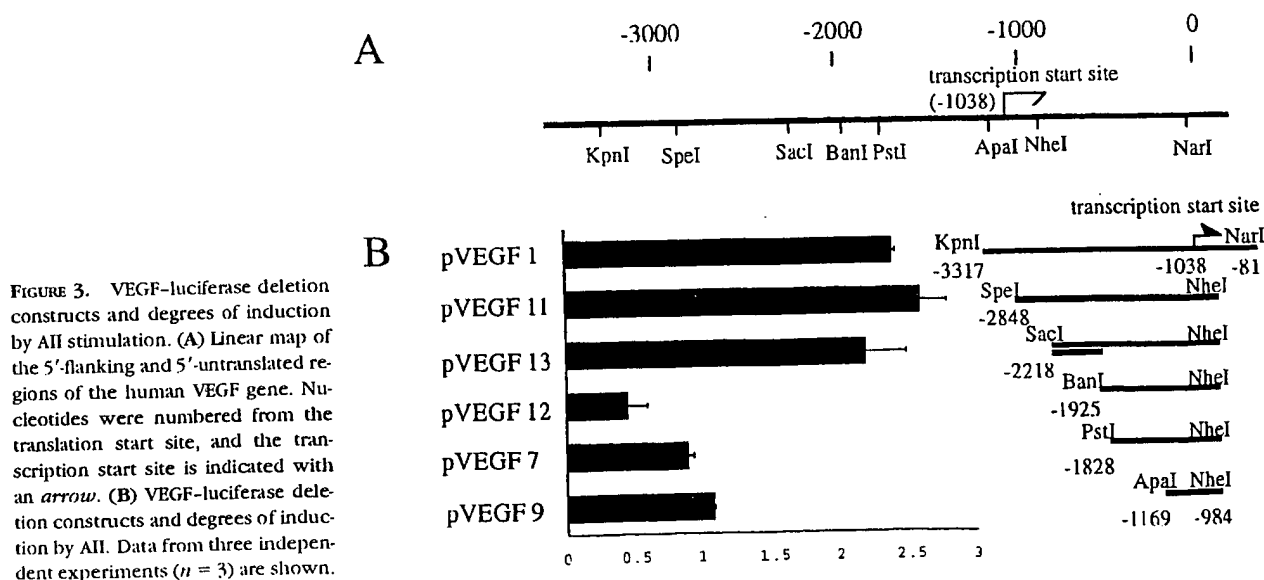


FIGURE 3. VEGF-luciferase deletion constructs and degrees of induction by AII stimulation. (A) Linear map of the 5'-flanking and 5'-untranslated regions of the human VEGF gene. Nucleotides were numbered from the translation start site, and the transcription start site is indicated with an arrow. (B) VEGF-luciferase deletion constructs and degrees of induction by AII. Data from three independent experiments ($n = 3$) are shown.

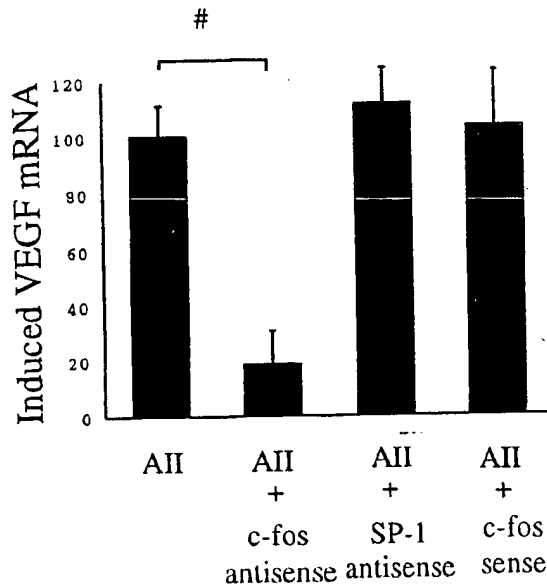


FIGURE 5. The effects of antisense oligonucleotides targeted against c-Fos and SP-1. BRP were pretreated with 5 μ M oligonucleotides for 8 hours before stimulation with AII for 4 hours, after which Northern blot analysis was performed. The induced VEGF mRNA under each condition is shown ($n = 3$, $\#P < 0.01$).

stimulation with AII. Antisense oligonucleotides against c-Fos blocked the AII-induced VEGF mRNA expression by $81.5 \pm 11.9\%$ ($P < 0.01$, Fig. 5). c-Fos sense and SP-1 antisense oligonucleotides did not affect AII-induced VEGF mRNA induction, and c-fos antisense oligonucleotides did not affect the basal VEGF synthesis (data not shown).

Increase of VEGF Protein Synthesis by AII

Bands at approximately 23 and 21 kDa were detected by immunoprecipitation with a rabbit anti-human VEGF antibody, and these are related to VEGF isoforms 165 and 121, respectively.⁴³ The major band, which represents VEGF 165, was increased 3.7 ± 0.6 -fold by AII stimulation at 10 nM (Fig. 6).

Growth Effect of Conditioned Medium from AII-Treated BRPs on BRECs

From five independent assays, conditioned media of the AII-treated BRPs increased the proliferation of BRECs 1.5 ± 0.1 -fold above control media levels ($P < 0.01$), and this effect was inhibited almost completely by adding VEGF neutralizing antibody (R&D Systems, Minneapolis, MN; Fig. 7).

DISCUSSION

Pericytes are intramural cells that surround endothelial cells in capillaries and postcapillary venules and have multiple physiologic functions, including regulation of vascular tone, vascular permeability, and endothelial growth and differentiation⁴⁶⁻⁴⁸ in retinal microvessels. Pericytes are the cells most proximal to endothelial cells and are in intimate contact via adhesion plaques, gap junctions, and pericytic processes.⁴⁹ The ratio of pericytes to endothelial cells is higher in the retina than in other tissues, suggesting that interaction between pericytes

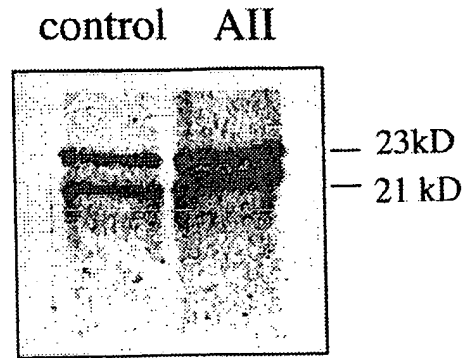


FIGURE 6. Immunoprecipitation analysis of AII-stimulated VEGF protein synthesis. BRPs were treated with AII (10 nM) or vehicle for 24 hours and labeled with ³⁵S-methionine. The cell lysates were incubated with a specific VEGF antibody and then immunoprecipitated with protein A Sepharose. Labeled proteins were visualized and analyzed using a densitometer. Three experiments ($n = 3$) were performed, and representative data are shown.

and endothelial cells is important in the retinal microcirculation. To the best of our knowledge, this study is the first to demonstrate that AII induces VEGF in retinal microcapillary

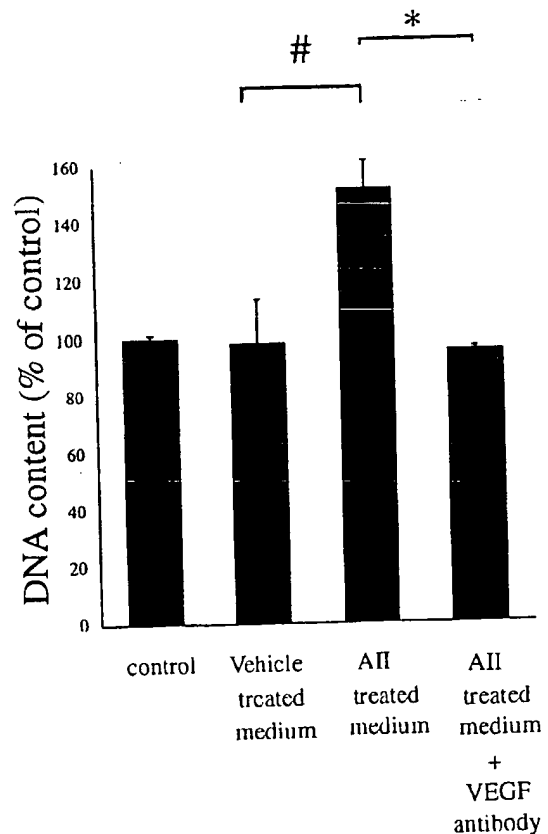


FIGURE 7. Conditioned media from AII-treated BRPs have growth-promoting effect on BRECs. BRPs were treated with AII (10 nM) or vehicle for 24 hours, and BRECs were cultured in the conditioned medium for 4 days. Cells were lysed, and DNA concentrations in each well were measured ($n = 5$, $\#P < 0.01$, $*P < 0.01$).

pericytes and that VEGF released by the pericytes stimulates retinal endothelial growth in a paracrine manner. We have demonstrated that AII significantly increases the level of VEGF mRNA in a time- and dose-dependent manner. We also detected the new protein synthesis of VEGF protein by immunoprecipitation assay (Fig. 6). In contrast to our results, a lack of significant effect of AII on VEGF expression was recently reported in bovine retinal pericytes.⁵⁰ In that report, the authors derived conclusions from the experiments using a single dose of 1 μ M AII. Our dose-dependent study revealed that peak response was observed at 10 nM, and the higher concentrations had less stimulatory effect on VEGF expression. The discrepancy probably results from the lack of dose-dependent experiments in that study. The significant response was observed at concentrations higher than normal circulating levels (1 nM). It is likely that local concentrations of AII in retinal microvasculature are much higher than serum and vitreous levels, because an autocrine-paracrine production system for AII is present in ocular tissues.²²

AII has two major receptor subtypes, AT₁ and AT₂.⁵¹ Most of the actions of AII are mediated by AT₁, but actions of the AT₂ are not well understood.¹⁴ In the present study, the effect of AII on VEGF expression was completely inhibited by the AT₁ receptor antagonist but not by the AT₂ antagonist, suggesting that AII-induced VEGF expression is mediated by AT₁ receptors. Although not to a significant degree, the average VEGF expression was increased by AT₂ receptor blockage. This might suggest that AT₂ receptors mediate an inhibitory effect on VEGF induction, which is in agreement with previous reports.^{52,53} However, further study is needed on the distribution of AII receptors and the changes in the AII effect on retinal microvascular systems.

The AII-induced increase in VEGF mRNA was rapid and peaked at 4 hours (Fig. 1A). Experiments in which actinomycin D was used to inhibit RNA synthesis indicate that the half-life of VEGF mRNA is 2.1 hours, which is in concordance with previous reports in SMCs,⁵⁴ and AII did not primarily change the mRNA stability of VEGF (Fig. 2). This suggests that AII-induced VEGF mRNA induction is most likely through transcriptional regulation. To further investigate transcriptional regulation of the VEGF gene, we performed transient transfection reporter assay using a series of deletion constructs of the 5'-flanking region of the human VEGF gene.^{42,43} As expected, reporter gene activities were upregulated by AII. In addition, we found that a 293-bp fragment (*SacI*-*BanI*) of the VEGF gene has a responsible element for AII stimulation (Fig. 3). Angiotensin II is reported to stimulate the expression of *c-fos* and *c-jun* and their respective proteins, c-Fos and c-Jun, which constitute the heterodimer complex called AP-1, which transactivates many genes that have a TPA responsive element (TRE) in their promoter region.⁵⁵ Because the AII-responsive region we found contains potential binding sites for AP-1, we further investigated a role for AP-1 in the induction of VEGF in BRPs. Northern blot analysis revealed rapid and marked *c-jun* and *c-fos* induction by AII in BRPs (Fig. 4), and pretreatment with *c-fos* antisense oligonucleotides blocked the AII-induced VEGF mRNA expression (Fig. 5). SP-1 antisense and *c-fos* sense oligonucleotides did not affect AII-induced VEGF expression. These data might suggest a predominant role of AP-1 and its TRE activation in AII induction of VEGF in BRPs.

To investigate AII effects on the retinal pericyte-endothelial cell paracrine system, we determined growth-promoting

effects of conditioned media from AII-treated pericyte cultures on BRECs. Conditioned media from AII-treated BRPs had a significantly greater stimulatory effect on BREC proliferation than did the media from unstimulated BRPs. As we reported previously, AII itself had no significant effect on BREC proliferation.²⁹ These data suggest that AII induces a paracrine molecule in BRPs, which activates endothelial cell proliferation. Angiotensin II has been reported to regulate the induction of several autocrine growth factors, such as platelet-derived growth factor (PDGF) A-chain, transforming growth factor- β (TGF- β), basic fibroblast growth factor (bFGF), and insulin-like growth factor I (IGF I).¹⁴⁻¹⁷ We did not examine the effect of AII on the regulation of these growth factors in BRPs; however, the addition of VEGF neutralizing antibody almost completely abolished growth stimulatory capacity of the conditioned media (Fig. 7). This observation suggests that VEGF is probably a predominant factor that mediates paracrine activation of endothelial cell growth.

It has been suggested that the contact between pericytes and endothelial cells caused inhibition of endothelial cell growth.⁴⁸ However, our observation that VEGF, which was produced in pericytes, induced endothelial cell growth in a paracrine manner indicates a proliferative effect of pericytes. In vivo, pericytes may have both effects, the balance of which is important for controlling endothelial cell growth. Under normal conditions, pericytes suppress endothelial cell growth by contact with endothelial cells, but in the later stage of diabetic retinopathy, when thickening of the basement membrane suppresses the contact, the inhibitory effect is overcome by its stimulatory effects. Our data suggest that RAS might be one of the important factors regulating this growth-promoting effect. Because pericyte loss is very advanced in the later stages of diabetic retinopathy, pericytes might contribute little to VEGF activity. However, VEGF has been shown to have a possible role in the early stages of retinopathy. This paracrine action of VEGF produced by pericytes might be more important in the early stages.

Together with our previous finding that AII potentiates VEGF-induced angiogenic activity through upregulation of VEGF receptor in retinal endothelial cells,²⁹ the present study further clarifies the role of RAS in the development of diabetic retinopathy. Angiotensin II has a prominent stimulatory effect not only on VEGF receptor expression in endothelial cells but also on VEGF production in pericytes in the retinal microcirculation. Further studies, including an in vivo study to see the effect of AT₁ antagonist, will strengthen this hypothesis.

In therapeutic aspects, inhibition of RAS is thought to be beneficial for the treatment of diabetic retinopathy. Indeed, the beneficial effects of ACE inhibition in patients with diabetic retinopathy have recently been shown in the EUCLID study and other studies.^{23,24,56} Our studies revealed that AT₁ receptor mediation is predominant for the AII-induced responses. An AT₁ blocker and ACE inhibitors might effectively prevent diabetic retinopathy.

References

1. Moss SE, Klein R, Klein BE. The incidence of vision loss in a diabetic population. *Ophthalmology*. 1988;95:1340-1348.
2. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-1309.

3. Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell*. 1992;3:211-220.
4. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. 1992;359:845-848.
5. Brock TA, Dvorak HF, Senger DR. Tumor-secreted vascular permeability factor increases cytosolic Ca^{2+} and von Willebrand factor release in human endothelial cells. *Am J Pathol*. 1991;138:213-221.
6. Clauss M, Gerlach M, Gerlach H, et al. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med*. 1990;172:1535-1545.
7. Tolentino MJ, Miller JW, Gragoudas ES, et al. Intravitreal injections of vascular endothelial growth factor produce retinal ischemia and microangiopathy in an adult primate. *Ophthalmology*. 1996;103:1820-1828.
8. Amin RH, Frank RN, Kennedy A, Elliott D, Puklin JE, Abrams GW. Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with nonproliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 1997;38:36-47.
9. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 1992;359:843-845.
10. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci USA*. 1995;92:10457-10461.
11. Adamis AP, Shima DT, Tolentino MJ, et al. Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. *Arch Ophthalmol*. 1996;114:66-71.
12. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994;331:1480-1487.
13. Adamis AP, Miller JW, Bernal MT, et al. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol*. 1994;118:445-450.
14. Dzau VJ. Molecular biology of angiotensin II biosynthesis and receptors. *Can J Cardiol*. 1995;11:21F-26F.
15. Daemen MJ, Lombardi DM, Bosman FT, Schwartz SM. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ Res*. 1991;68:450-456.
16. Naftilan AJ, Pratt RE, Dzau VJ. Induction of platelet-derived growth factor A-chain and c-myc gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest*. 1989;83:1419-1424.
17. Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *J Clin Invest*. 1993;91:2268-2274.
18. Delafontaine P, Lou H. Angiotensin II regulates insulin-like growth factor I gene expression in vascular smooth muscle cells. *J Biol Chem*. 1993;268:16866-16870.
19. Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. *Hypertension*. 1992;19:753-757.
20. Feman SS, Mericle RA, Reed GW, May JM, Workman RJ. Serum angiotensin converting enzyme in diabetic patients. *Am J Med Sci*. 1993;305:280-284.
21. Danser AH, van den Dorpel MA, Deinum J, et al. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J Clin Endocrinol Metab*. 1989;68:160-167.
22. Danser AH, Derckx FH, Admiraal PJ, Deinum J, de Jong PT, Schalekamp MA. Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci*. 1994;35:1008-1018.
23. Jackson WE, Holmes DL, Garg SK, Harris S, Chase HP. Angiotensin-converting enzyme inhibitor therapy and diabetic retinopathy. *Ann Ophthalmol*. 1992;24:99-103.
24. Parving HH. Impact of blood pressure and antihypertensive treatment on incipient and overt nephropathy, retinopathy, and endothelial permeability in diabetes mellitus. *Diabetes Care*. 1991;14:260-269.
25. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science*. 1992;255:989-991.
26. Millauer B, Witzigmann Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*. 1993;72:835-846.
27. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA*. 1993;90:7533-7537.
28. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*. 1998;92:735-745.
29. Otani A, Takagi H, Suzuma K, Honda Y. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res*. 1998;82:619-628.
30. Williams B, Baker AQ, Gallacher B, Lodwick D. Angiotensin II increases vascular permeability factor gene expression by human vascular smooth muscle cells. *Hypertension*. 1995;25:913-917.
31. Chua CC, Handy RC, Chua BH. Upregulation of vascular endothelial growth factor by angiotensin II in rat heart endothelial cells. *Biochim Biophys Acta*. 1998;1401:187-194.
32. King GL, Goodman AD, Buzney S, Moses A, Kahn CR. Receptors and growth-promoting effects of insulin and insulinlike growth factors on cells from bovine retinal capillaries and aorta. *J Clin Invest*. 1985;75:1028-1036.
33. Gitlin JD, D'Amore PA. Culture of retinal capillary cells using selective growth media. *Microvasc Res*. 1983;26:74-80.
34. Wong PC, Hart SD, Zaspel AM, et al. Functional studies of non-peptide angiotensin II receptor subtype-specific ligands: Dup753 (AII-1) and PD123177 (AII-2). *J Pharmacol Exp Ther*. 1990;255:584-592.
35. Timmermans PBMW, Wong PC, Chiu AT, Herblin JAM, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev*. 1993;45:205-251.
36. Nayak RC, Berman AB, George KL, Eisenbarth GS, King GL. A monoclonal antibody (3G5)-defined ganglioside antigen is expressed on the cell surface of microvascular pericytes. *J Exp Med*. 1988;167:1003-1015.
37. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156-159.
38. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*. 1992;257:967-971.
39. Takagi H, King GL, Ferrara N, Aiello LP. Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. *Invest Ophthalmol Vis Sci*. 1996;37:1311-1321.
40. van Straaten F, Muller R, Curran T, Van Beveren C, Verma IM. Complete nucleotide sequence of a human c-onc gene: deduced amino acid sequence of the human c-fos protein. *Proc Natl Acad Sci USA*. 1983;80:3183-3187.
41. Angel P, Allegretto EA, Okino ST, et al. Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. *Nature*. 1988;332:166-171.
42. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J Biol Chem*. 1991;266:11947-11954.
43. Ikeda E, Achen MG, Breier G, Risau W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem*. 1995;270:19761-19766.
44. Yoshida S, Ono M, Shono T, et al. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth

- factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol Cell Biol.* 1997;17:4015-4023.
45. Thieme H, Aiello LP, Takagi H, Ferrara N, King GL. Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes.* 1995;44:98-103.
46. Kelley C, D'Amore P, Hechtman HB, Shepro D. Vasoactive hormones and cAMP affect pericyte contraction and stress fibres in vitro. *J Muscle Res Cell Motil.* 1988;9:184-194.
47. Shepro D, Morel NM. Pericyte physiology. *FASEB J.* 1993;7:1031-1038.
48. Orlidge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol.* 1987;105:1455-1462.
49. Larson DM, Carson MP, Haudenschild CC. Junctional transfer of small molecules in cultured bovine brain microvascular endothelial cells and pericytes. *Microvasc Res.* 1987;34:184-199.
50. Kim Y, Imdad RY, Stephenson AH, Sprague RS, Lonigro AJ. Vascular endothelial growth factor mRNA in pericytes is upregulated by phorbol myristate acetate. *Hypertension.* 1998;31:511-515.
51. Whitebread S, Mele M, Kamber B, de Gasparo M. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun.* 1989;163:284-291.
52. Nakajima M, Hutchinson HG, Fujinaga M, et al. The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci USA.* 1995;92:10663-10667.
53. Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest.* 1995;95:651-657.
54. Li J, Perrella MA, Tsai JC, et al. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. *J Biol Chem.* 1995;270:308-312.
55. Curran T, Franza B Jr. Fos and Jun: the AP-1 connection. *Cell.* 1988;55:395-397.
56. Chaturvedi N, Sjolie AK, Stephenson JM, et al. Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes: the EUCLID Study Group—EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus. *Lancet.* 1997;351:28-31.



Early contribution of pericytes to angiogenic sprouting and tube formation*

Ugur Ozerdem¹ & William B. Stallcup²

¹La Jolla Institute for Molecular Medicine, Vascular Biology Division, La Jolla, California, USA; ²The Burnham Institute, Neurobiology Program, La Jolla, California, USA

Received 7 August 2003; accepted in revised form 30 November 2003

Key words: angiogenesis, endothelium, neovascularization, NG2, PDGF β -receptor, pericyte, sprout, tube, tumor, transplantation

Abstract

Immunostaining with endothelial and pericyte markers was used to evaluate the cellular composition of angiogenic sprouts in several types of tumors and in the developing retina. Confocal microscopy revealed that, in addition to conventional endothelial tubes heavily invested by pericytes, all tissues contained small populations of endothelium-free pericyte tubes in which nerve/glial antigen 2 (NG2) positive, platelet-derived growth factor beta (PDGF β) receptor-positive perivascular cells formed the lumen of the microvessel. Perfusion of tumor-bearing mice with FITC-dextran, followed by immunohistochemical staining of tumor vasculature, demonstrated direct apposition of pericytes to FITC-dextran in the lumen, confirming functional connection of the pericyte tube to the circulation. Transplantation of prostate and mammary tumor fragments into NG2-null mice led to the formation of tumor microvasculature that was invariably NG2-negative, demonstrating that pericytes associated with tumor microvessels are derived from the host rather than from the conversion of tumor cells to a pericyte phenotype. The existence of pericyte tubes reflects the early participation of pericytes in the process of angiogenic sprouting. The ability to study these precocious contributions of pericytes to neovascularization depends heavily on the use of NG2 and PDGF β -receptor as reliable early markers for activated pericytes.

Abbreviations: BrdU – bromodeoxyuridine; CD31 – PECAM-1; CD105 – endoglin; flk 1 – VEGF receptor-2; LNCaP – prostatic carcinoma cell line derived from a supraclavicular lymph node metastasis; NG2 – nerve/glial antigen 2; PBS – phosphate-buffered saline; PC-3 – prostatic carcinoma cell line derived from a bone metastasis of a patient; PDGF β -receptor – platelet-derived growth factor beta receptor; α -SMA – alpha isoform of smooth muscle actin; TRAMP – transgenic adenocarcinoma of mouse prostate

Introduction

Angiogenesis is essential for almost all aspects of normal development, as well as for many pathological processes, including tumor growth and metastasis [1, 2]. The walls of typical angiogenic microvessels are composed of two principal cell types: endothelial cells, which form the inner lining of the vascular tube, and pericytes (mural cells) which form an outer sheath around the endothelium [3, 4]. While endothelial cells have been studied extensively, much less is known about the pericyte, a name ('peri' around and 'cyto' cell) that denotes the cell's periendothelial location at the abluminal aspect of

microvessels [5]. A recent search of the PUBMED database at <http://www.ncbi.nlm.nih.gov> reveals a 118-fold difference between the number of papers published on these two vascular cell types. Since the cellular processes underlying neovascular sprout formation remain incompletely understood [6, 7], increased attention to pericytes and their interaction with endothelial cells will be required not only to attain a better understanding of vascular biology, but also to realize the full potential of anti-angiogenic therapy in oncology.

The relationship between endothelial cells and pericytes varies from tissue to tissue. As one example, the density of pericyte investment of the microvascular endothelium is quite high in the developing central nervous system, but very low in skeletal muscle [8, 9]. In tumor neovascularity this relationship may be even more variable. Underscoring the heterogeneity of tumor vasculature, as many as nine distinct angiogenic vessel classes have been described in neoplasms, based on both morphology and the cellular composition of the vessel

* Presented in part at the vascular biology symposium of Federation of American Societies for Experimental Biology (FASEB), 2003, Annual Meeting in San Diego, California, USA.

Correspondence to: Ugur Ozerdem, MD, Assistant Professor, La Jolla Institute for Molecular Medicine, Vascular Biology Division, 4570 Executive Drive, Suite 100, La Jolla, CA 92037, USA. Tel: +1-858-587-8788; Fax: +1-858-587-6742; E-mail: ozerdem@ljim.org

Table 1. The varieties and classification of blood vessels in the substance of neoplasm. (From Warren BA [10]).

Class 1. Arteries and arterioles
Class 2. Capillaries
Class 3. Capillary sprouts
Class 4. Sinusoidal vessels
Class 5. Blood channels without endothelial lining
Class 6. Giant capillaries
Class 7. Capillaries with fenestrated endothelium
Class 8. Venules and veins
Class 9. Arterio-venous anastomoses

wall (Table 1) [10]. One of these vessel classes (class 5) describes vascular walls in which the endothelium is discontinuous. Examples of class-5 vessels have recently become more numerous with the description of mosaic vessels [11, 12] and the phenomenon of vasculogenic mimicry [13–15].

These vessel types call to mind a recent suggestion from our studies that vascular pericytes can contribute to the heterogeneous nature of microvessels, even in non-tumor situations. In a model of corneal angiogenesis, we found endothelial cell-free segments of microvessels in which pericytes appeared to comprise the lumen of the microvascular tube [16]. In the current study we further analyze the ontogeny, morphology, and functional properties of pericyte tubes in normal retinal vasculature and in several types of tumors (melanoma, mammary, prostate, lung, glioma). This study expands our understanding of the contribution of pericytes to the process of angiogenic sprouting, demonstrating the early participation of these perivascular cells in microvascular tube formation.

Materials and methods

Tumors

Lewis lung carcinoma [17] and B16F1 melanoma [18] tumors resulted from subcutaneous injection of 5×10^6 cells into C57Bl/6 mice. Human prostatic carcinoma cell line derived from a bone metastasis of a patient (PC-3) prostate [19], prostatic carcinoma cell line derived from a supraclavicular lymph node (LNCaP) prostate [20] and U87MG glioma tumors [21] were grown subcutaneously in athymic (Crl nu:nu) mice. Mammary tumors were obtained from female MMTV/PyMT mice, which spontaneously develop neoplasms due to activation of the polyoma middle T oncogene (MT) under control of the mouse mammary tumor virus (MMTV) promoter [22]. Prostate tumors were obtained from transgenic adenoma of prostate (TRAMP) mice [23].

Retinal vascular development

Vascular development in the retina progresses from the optic nerve head, extending peripherally along the inner retinal surface and finally invading the outer retinal

layers. The laminar anatomy of the retina and the fact that vascularization largely occurs postnatally make this tissue a very useful model for the study of neovascular sprouting [24, 25]. Eyes were taken from postnatal days 2 and 7 (P2 and P7) C57Bl/6 mice following daily intraperitoneal bromodeoxyuridine (BrdU) (Sigma, St. Louis, Missouri) injections (80 μ g/g body weight) to allow subsequent identification of proliferating vascular cells. Eyes were sectioned in a plane oriented sagittally to the optic nerve, so that each section represented a slice of the entire eyeball and retina. Immunostaining was then used to identify the tips of the most peripheral blood vessels in the primary vascular plexus, marking the transition between the vascular and avascular retinal tissue.

Transplantation of transgenic prostate and breast tumors

Breast and prostate tumors, respectively, were dissected from sacrificed MMTV/PyMT and TRAMP mice, both of which have an nerve/glial antigen 2 (NG2) +/+ , C57Bl/6 genetic background. As described below, tumor fragments were transplanted into C57Bl/6, NG2-/- (NG2 knockout) mice [26] to determine whether the pericytes in tumor neovasculature are host or tumor-derived.

For mammary tumor transplantation, female NG2 knockout mice were anesthetized with Avertin, and an inverted Y-shaped incision was made through the abdominal skin [27]. The number-four mammary fat pads were exposed by a blunt dissection, and a small incision (1 mm) was made in each fat pad to accommodate 1 mm³ PyMT tumor fragments. The incision was closed using metal clips, and the mice were followed for 3 months to allow tumors to grow to a size of 1 cm³.

The cornea is a transparent, avascular tissue which enables real-time identification of new pathologic vessels that form as a result of implantation of tumor fragments [28]. NG2 knockout mice were anesthetized with Avertin, and tumor fragments (0.027 mm³) from 8-month-old TRAMP mice were implanted into corneal stromal micropockets created using a modified von Graefe knife [29]. The mice were monitored by biomicroscopic examination for two weeks to follow the progress of tumor vascularization within the corneal stroma.

Immunohistochemistry and vascular imaging

Following fixation of all tissues in 4% paraformaldehyde, cryopreservation in 20% sucrose in phosphate-buffered saline (PBS) at 4 °C, embedding in O.C.T. compound (Sakura Inc., Torrance, California), and snap-freezing, frozen histologic sections (40–80 μ m) were cut using a Reichert cryostat (Reichert Inc., Buffalo, New York).

Endothelial cells have been shown to express different cell surface markers as a function of developmental age [30]. We therefore identified endothelial cells using a cocktail of antibodies against endoglin (CD105).

PECAM-1 (CD31), and VEGF receptor-2 (flk-1) (Pharmingen, San Diego, California), a strategy that has been previously utilized to maximize labeling of all vascular endothelial cells, both immature and mature [11]. Pericytes were identified by labeling with affinity-purified rabbit polyclonal antibodies prepared in our laboratory against the NG2 proteoglycan or the platelet-derived growth factor beta receptor (PDGF β -receptor) [9, 16]. Both NG2 and PDGF β -receptor are regarded as specific markers for pericytes [12, 31]. Proliferative pericyte and endothelial cell populations were identified immunohistochemically using anti-BrdU antibody (Fitzgerald Industries, Concord, Massachusetts) [32-34]. Briefly, frozen sections were digested with 0.005% pepsin (Sigma, St. Louis, Missouri) in 0.01 HCl for 30 min at 37 °C followed by treatment with 4N HCl for 30 min at room temperature. Sections were then blocked by incubation in 5% goat serum in PBS for 30 min [35] prior to incubation with antibody.

Confocal microscopic imaging of combined endothelial (CD31 + CD105 + VEGF receptor-2, flk-1) [11, 30] and pericyte markers (NG2 or PDGF β -receptor) was performed as described previously [9, 16, 36]. Briefly, optical sections were obtained from the specimens using the Bio-Rad MRC-1024MP confocal microscope. Serial optical sections (1 μ m each) across the entire thickness (40-80 μ m) of the histological specimens were overlaid (Z-stack) to provide reconstructions of entire vessels. This allowed unambiguous identification of the spatial relationship between pericytes and endothelial cells in the vessel wall. In order to estimate the frequency of vessels in any given tissue that contained endothelium-free segments (pericyte tubes), we performed systematic random sampling [37] to obtain 10 histologic sections for each tissue type to represent the corresponding frozen tissue block. Following immunohistochemistry, we counted the number of such endothelium-free segments (pericyte tubes) by microscopy, and divided the number of pericyte tubes by the total number of vessels in immunostained sections.

Fluorescein microangiography integrated with immunohistochemistry

To determine the functionality of pericyte tubes in a subcutaneous B16F1 melanoma, we performed fluorescein angiography using high molecular weight (two million daltons) dextran conjugated with fluorescein isothiocyanate (Sigma, St. Louis, Missouri). Since FITC-dextran remains completely within the vascular lumen without diffusion or decay [38], this procedure identifies only those vessels that are actively perfused by the circulation. FITC-dextran-perfused tissues were sectioned and immunostained for PDGF β -receptor or NG2 (red fluorochrome) to identify vessel walls in which pericytes interface directly with the intraluminal FITC-dextran.

Results

Pericyte tubes in early angiogenic sprouts

Examination of sections taken from spontaneous mammary tumors in female MMTV-PyMT mice reveals that a subpopulation of angiogenic tumor microvessels (1%) contain endothelium-free segments in which the vascular lumen is formed by a pericyte tube (Figures 1a-c). This phenomenon is remarkably similar to that observed in our corneal angiogenesis studies [16]. The Z-stack overlay demonstrates the absence of endothelial markers associated with the pericyte tube across the entire extent of the vessel in question (arrows and Δ in panel c). In the more typical vessels containing endothelial elements, we consistently noted extensive pericyte investment of the endothelium (panel c). Since it has been proposed that activated pericytes may be involved in creating/marking pathways for invading endothelial cells [4, 39], we wondered if pericyte tubes might represent an early developmental stage of microvessel development at which formation of the vascular endothelium is not complete. To test this possibility, we examined much younger (12 days old) tumors produced by subcutaneous grafts of mouse Lewis lung carcinoma cells in C57Bl/6 mice. Sections of these tumors contain even more striking examples in which entire vessels appear to be composed of pericyte tubes (Figures 1d-f). As before, the Z-stack demonstrates that these tubes are devoid of endothelial markers across their entire width. Even younger tumors (7 days) produced by subcutaneous xenografts of human PC-3 prostate tumor cells into athymic male mice (Figures 1g-i) contain examples not only of endothelium-free pericyte tubes (arrows and Δ in panel i), but also large numbers of individual pericytes invading the tumor in the absence of endothelial cells. TRAMP, LNCaP, and U87MG glioma tumors were also examined and found to contain populations of pericyte tubes (data not shown).

To investigate the occurrence of pericyte tubes in normally developing microvasculature, we examined the mouse retina, in which a primary vascular plexus develops postnatally at the interface between the retina and vitreous. In addition to comparing endothelial and pericyte-specific markers, we also evaluated these samples for cell proliferation by immunohistochemical staining for BrdU incorporation. The sagittal orientation of the sections made it possible to identify the tips of angiogenic sprouts growing toward more peripheral regions of the retina (arrow in Figure 2a). We used Nomarski interference microscopy (as in Figure 2c) to help choose sections that appeared to contain intact sprout tips. Between postnatal days 2 and 7 it is clear that pericytes and endothelial cells are both present at the growing tip of the vascular sprout. Using confocal microscopy, we found examples of endothelium-free segments of growing sprouts that appeared to be formed by pericytes (Δ in panel b), demonstrating the occurrence of pericyte tubes in normal as well as pathological

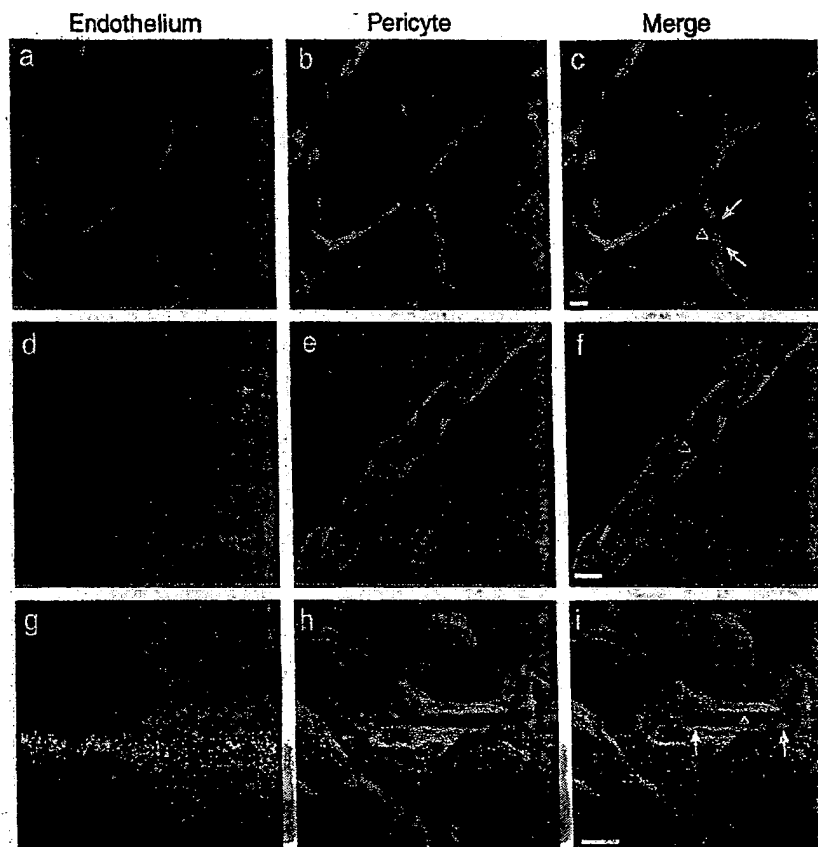


Figure 1. Pericyte tubes are a component of tumor microvasculature. Frozen sections were taken from spontaneous MMTV-PyMT breast carcinoma (a–c), subcutaneous Lewis lung carcinoma (d–f), and subcutaneous PC-3 prostate tumor (g–i). Vascular endothelial elements (red) are identified by combined immunohistochemical staining for CD31, CD105, and flk-1. Pericytes (green) are identified by staining for NG2 proteoglycan. All panels are Z-stack confocal images. All three tumor types contain endothelium-free pericyte tubes, labeled by Δ in the merged images of panels c, f and i. The margins of complete pericyte tubes are indicated with arrows in c and i. In f the entire segment is a pure pericyte tube. Note the absence of endothelium (a, d, g and c, f, i) in regions that contain pericyte tubes (b, e, h). Scale bars indicate 10 μ m.

microvasculature. The frequency of pericyte tubes in retinal microvasculature was similar to that seen in the various tumor models (1%). Strikingly, both endothelial cells (arrows) and pericytes (arrowheads) in growing retinal vessel tips have nuclei that are BrdU-positive. The pericyte nucleus characteristically fills almost the entire soma of the cell and bulges away from the lumen of the microvessel [8]. These factors explain why some pericyte nuclei in Figure 2b occupy an extreme peripheral position relative to the vessel. It is clear from the accompanying Nomarski image (Figure 2c) however, that these nuclei belong to pericytes that are closely associated with the microvessel. The presence of these BrdU-labeled nuclei is consistent with the nascent character of the vessel and shows that both cell populations are mitotically active at this stage. In microvessels such as these that contain both pericytes and endothelial cells, we always noted extensive investment of the endothelium by PDGF β -receptor-positive, NG2-positive pericytes. This is a general characteristic of microvessels in the central nervous system [3, 8, 31].

Pericyte tubes are perfused by the tumor circulation

Sections of a subcutaneous B16F1 melanoma tumor perfused with FITC-dextran were examined to evaluate whether pericyte tubes are a functional component of the circulation. In 1% of the angiogenic vessels in these specimens, we observed a direct interface (Figure 3c) between pericytes (Figure 3a) and luminal FITC-dextran (Figure 3b), indicative of patency of the pericyte tube. This phenomenon was seen using either PDGF β -receptor immunostaining (Figure 3) or NG2 immunostaining (not shown) to identify pericytes. Since FITC-dextran does not leak and diffuse from blood vessels, the only way for a pericyte tube to be filled with the dye is to be connected to the active circulation.

Pericytes in transplanted tumor microvessels are of host origin

Just as it has been suggested that tumor cells can display endothelial markers and contribute to forming the

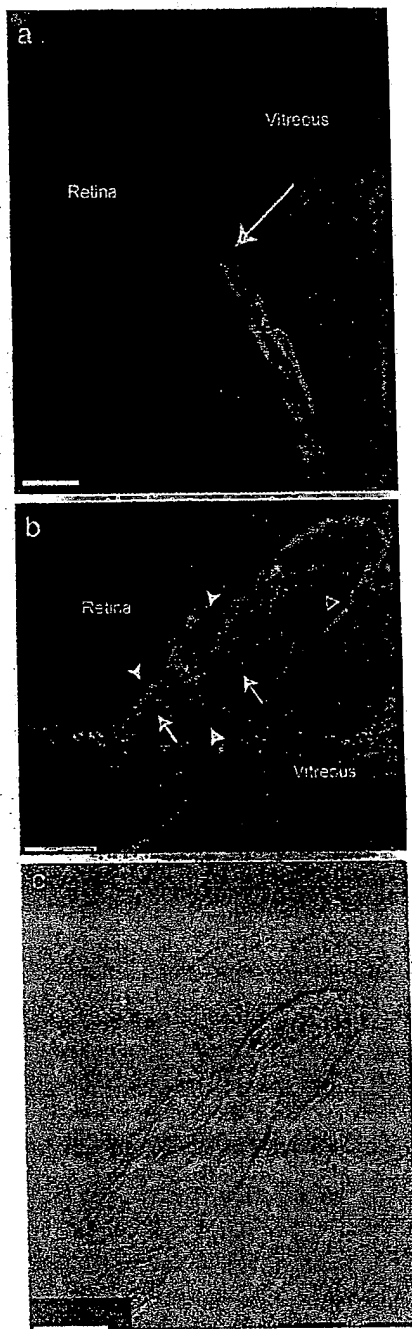


Figure 2. Early pericyte association with angiogenic microvessels in newborn mouse retina. Frozen sections of P2 (a) and P7 (b and c) mouse retina were processed by immunohistochemistry to identify the vascular endothelium (combined CD31 + CD105 + β -tub, red), pericytes (NG2, green), and nuclei of mitotic cells (BrdU, purple, panel b). Z-stack confocal images show that vascular pericytes extensively invest the angiogenic sprouts at both ages. Arrow in panel a indicates the tip of the growing angiogenic sprout at the vascular/avascular junction in the P2 peripheral retina. At P7 (panels b and c) both pericytes (arrowheads) and endothelial cells (arrows) contain BrdU-positive nuclei. Δ marks an endothelium free, pericyte tube-containing region of the angiogenic sprout. Panel c is the Nomarski image of a single focal plane from the specimen shown in panel b. Scale bars indicate 20 μ m (a) and 5 μ m (b, c).

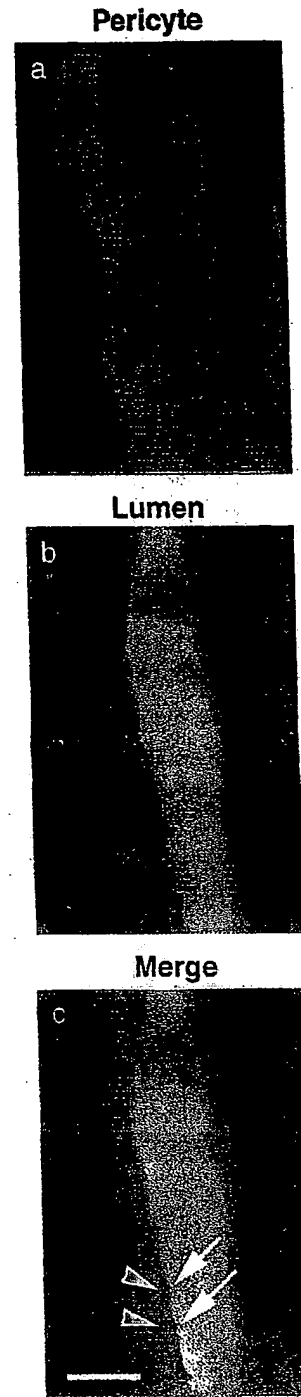


Figure 3. Functional (perfused) pericyte tube. In sections obtained from a subcutaneous B16F1 melanoma perfused with FITC-dextran, a pericyte tube immunostained (red) for PDGF β -receptor (a), is in direct contact with the vascular lumen filled with FITC-dextran (green) (b). In the merged image (c), arrowheads identify pericytes forming the vessel wall. Arrows indicate FITC-dextran in the lumen of the pericyte tube. The direct interface between these two labels precludes the presence of an intervening endothelial cell layer. Scale bar indicates 10 μ m.

lumen of tumor vessels, it is formally possible that our observations could be explained by the acquisition of pericyte markers (such as NG2 and PDGF β -receptor) by tumor cells at the vascular lumen. Arguing against this possibility, B16F1, PC3, Lewis lung carcinoma, LNCaP, and U87MG cells were all found to be negative for CD105, CD31, flk-1, NG2 and PDGF β -receptor expression both *in vivo* and *in vitro*. TRAMP and PyMT tumor cells were also negative for NG2 expression (Figures 4b, d and f). Still, this does not preclude the possibility that rare, specialized tumor cells at the vascular lumen might be induced to express NG2 by virtue of the novel environment. Tumor transplantation experiments were performed to address this possibility.

Analysis of breast tumor fragments transplanted from an NG2-positive (MMTV/PyMT) donor into the fat pads of an NG2 knockout host revealed PDGF β -receptor-positive pericytes associated with angiogenic sprouts invading the tumor from the periphery (Figure 4a). These pericytes were invariably NG2-negative (Figure 4b). Likewise, examination of prostate tumor fragments transplanted from an NG2-positive donor (TRAMP) into the cornea of an NG2 knockout host revealed PDGF β -receptor-positive pericytes (Figure 4c) associated with the corneal neovasculature. Once again, these pericytes were always negative for NG2 expression (Figure 4d). If the luminal NG2-positive cells we have observed in our experiments with NG2-wildtype mice were of tumor origin, we would also expect to find such cells in the tumor vasculature of NG2-null mice. The fact that PDGF β -receptor-positive luminal cells express NG2 in tumors grown in wildtype mice but not in tumors grown in NG2-null mice argues strongly that luminal pericytes of the tumor microvasculature are derived from the host rather than from any component of the donor tumor.

Discussion

Two of the observations presented in our study seem especially noteworthy. First, mitotically active pericytes are associated with angiogenic sprouts during the early phases of neovascularization in both pathological (tumors) and normal (retinal) tissues. Second, pericytes alone can invade tissues in the absence of endothelial cells and can form functional, endothelium-free tubes that may be sub-classified as 'pericyte tubes' under the category of class-5 vessels.

These observations support the idea that activated, mitotic pericytes play an early role in the development of angiogenic sprouts and vessels. Coupled with our previous finding of pericytes associated with angiogenic sprouts in the embryonic central nervous system and in postnatal neovasculature formed in response to ischemia or growth factors [9, 16], these results emphasize the early participation of pericytes in both physiological and pathological angiogenesis. Activated pericytes are present as early as mitotic endothelial cells, and in some

instances appear to take the lead in establishing not only pathways of invasion, but also the formation of actual tubes. These findings are not entirely consistent with data suggesting that pericytes have only a late role in angiogenesis [40–42], but instead provide support for reports concerning an early role for pericytes in the formation of angiogenic sprouts [4, 12, 31, 39, 43–47]. Our identification of perfused, endothelium-free pericyte tubes comprising up to 1% of the total number of microvessels in our various preparations may be a reflection of the kinetics of angiogenic development. While pericytes may have a leading role in establishing pathways of invasion and formation of angiogenic tubes, endothelial cell participation cannot lag far behind, and may in fact occur almost simultaneously. Thus in most cases, especially in normal tissues, we observe pericytes and endothelial cells working in concert to form angiogenic microvessels composed of endothelial tubes extensively invested by pericytes. In this sense, pericyte tubes and pericyte-invested endothelial tubes do not represent distinct functional entities, but instead different developmental stages of the same process. In only a small number of instances do we catch a glimpse of pericyte tubes that are not yet fully lined by endothelial cells. It seems possible that sufficient dysregulation could occur during tumor angiogenesis to render the early contributions of pericytes more easily detectable.

An alternative mechanism for the formation of pericyte tubes could be the loss of endothelial cells as part of the process of vascular regression/pruning and remodeling. We cannot definitively rule out this possibility. However, it seems the less likely of the two alternatives, based on the following observations. In the developing retina, neovascular remodeling is primarily seen in the central retina, rather than in peripheral areas of the retina that contain the newest angiogenic sprouts [48]. The examples shown in Figure 2 are taken from the peripheral retina at the outermost boundary between the vascular and avascular retina. The structures shown are therefore likely to be nascent sprouts rather than vessels undergoing remodeling. The presence of BrdU-positive endothelial cell nuclei further supports the idea that these are new sprouts rather than regressing vessels. In the tumor studies, it is more difficult to evaluate the ontogeny of vessels in the absence of the stereotypical tissue architecture found in the retina. On the basis of tumor age and the possible existence of hypoxic areas within the tumor, it seems possible that regressing vessels might contribute to the labeling pattern seen in the mature mammary tumors (Figures 1a–c). It seems much less likely that endothelial cell regression can explain the patterns seen in the small, nascent tumor xenografts examined only 12 (d–f) and 7 (g–i) days after tumor cell injection. Endothelial cells from regressing vascular segments usually do not disappear (for example via apoptosis), but instead are re-utilized for the formation of new vessels [49]. Thus, the observation of pericytes invading the seven-day-old PC-3 tumor and

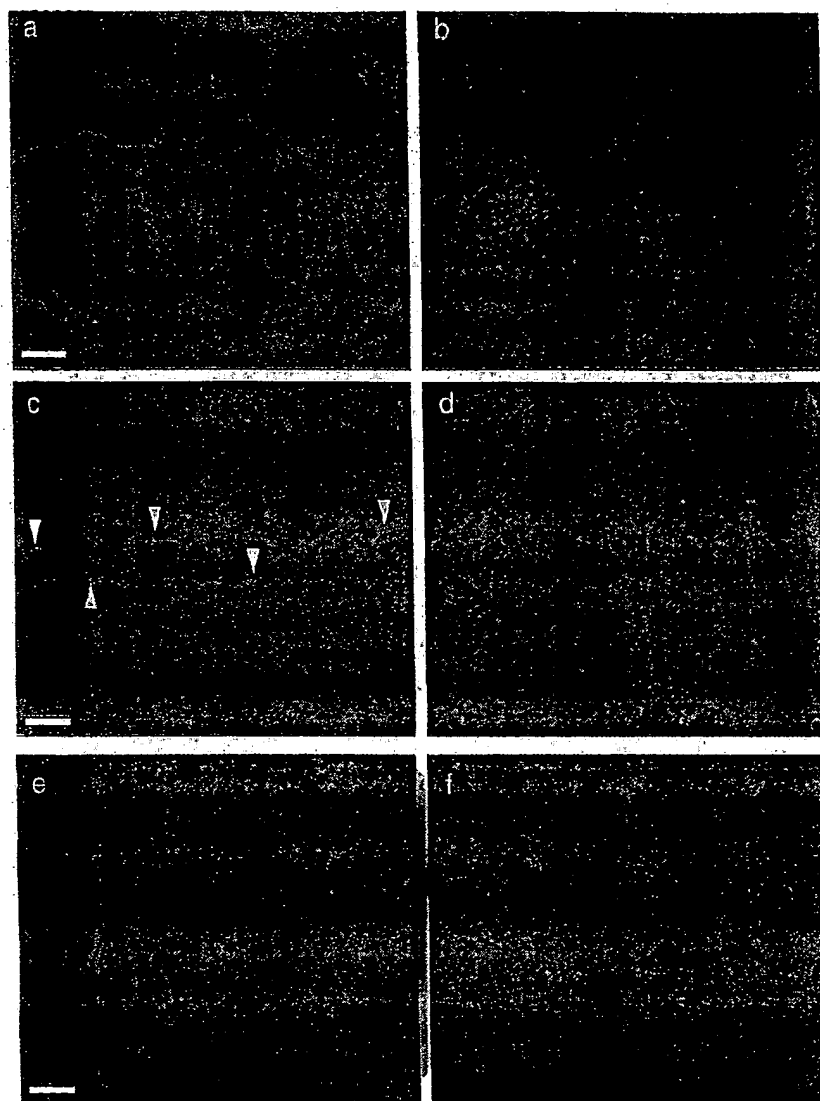


Figure 4. Host origin of pericytes in tumors. (a, b) Section from a PyMT breast tumor fragment transplanted to the fat pad of an NG2 knockout mouse is double-stained for PDGF β -receptor (a) and NG2 (b). Vascular pericytes invading the tumor are immunostained for PDGF β -receptor (a) but not for NG2 (b). (c, d) Section of a TRAMP prostate tumor fragment transplanted to the corneal stroma of an NG2 knockout mouse is double-stained for PDGF β -receptor (c) and NG2 (d). Neovascular pericytes are immunostained for PDGF β -receptor (c) but not for NG2 (d). Arrowheads indicate feeder vessels invading the cornea. (e, f) Section of PyMT breast tumor fragment transplanted to the fat pad of NG2 knockout mouse is double-stained for endothelial markers (CD105 + CD31 + flk1) (e) and NG2 (f). Angiogenic vessels exhibit immunostaining for endothelial markers (e) but not for NG2 (f). Tumor cells are not immunostained for either endothelial markers (e) or NG2 (f). Scale bars indicate 20 μ m.

forming tubes in the absence of endothelial cells is more suggestive of the formation of new microvessels than the regression of old ones.

Although pericytes are widely regarded to be the microvascular equivalent of smooth muscle cells, the origin, development, and function of these cells seem to be variable and complex [8, 50, 51]. Reliable identification of pericytes is important for understanding the role of these cells in angiogenic sprout formation and vascular heterogeneity. Our ability to detect the precocious contribution of pericytes to microvascular development depends heavily on the use of PDGF β -receptor and NG2 proteoglycan as markers for these activated

mural cells at an early stage of their development [12, 31]. One of the traditional markers for pericyte identification has been the expression of alpha-smooth muscle actin (α -SMA). However, a growing body of evidence suggests that α -SMA is a late marker for differentiated pericytes in rodents and therefore may be poorly expressed in developing angiogenic microvasculature [12, 31]. Since only a fraction of developing pericytes can be identified on the basis of α -SMA expression [4, 12, 52–55], the absence of immunoreactivity for α -SMA does not necessarily indicate the absence of pericytes. By comparison, NG2 is more widely expressed by pericytes, especially during early stages of development. Pericytes

expressing NG2 also express other pericyte markers such as PDGF β -receptor [9, 16, 36, 56–58] and aminopeptidase A [45]. Therefore, NG2 is an established, specific marker for identification of pericytes. It is expressed by pericytes in angiogenic microvasculature during pre- and postnatal development [9, 59], during tumor angiogenesis [43, 44, 60], in granulation tissue [43, 44, 58, 61], and in corneal and retinal angiogenesis models [16].

Our results more firmly establish endothelium-free pericyte tubes as a valid species of angiogenic vessel. Our tumor fragment transplantation experiments support previous results demonstrating a host origin for pericytes in tumor neovasculature [62]. Direct visualization of the invasion of host-derived, GFP-negative cells into GFP-positive tumors has provided additional evidence for the host origin of tumor vasculature [63]. Our combined fluorescein angiography-immunohistochemistry analysis of tumor vasculature is also of interest in this regard, since it provides an additional means of visualizing functional, newly formed pericyte tubes that have recently invaded the tumor.

In summary, our study provides evidence that pericytes contribute to the early phases of angiogenic sprout formation during both neoplastic and non-neoplastic neovascularization. The early participation of pericytes in microvascular development has important implications for therapeutic intervention in the many pathologies in which angiogenesis is a factor. As early players in angiogenesis, pericytes represent an additional target for treatments designed either to up-regulate (for example in ischemic disorders) or down-regulate (for example in cancer) vascularization. Recent studies suggest that dual targeting of pericytes and endothelial cells improves the efficacy of treatments aimed at the destruction of tumor masses [64, 65]. Clearly, the design of improved dual targeting strategies aimed at both pericytes and endothelial cells will depend on further understanding of the role of pericytes in neovascularization.

Acknowledgements

We are indebted to Larry Young, University of California at Davis, for providing the PyMT mammary tumor specimens. This work was supported by postdoctoral fellowship 0120036Y from the American Heart Association Western States Affiliate, New Investigator Grant PC020822 from the US Department of Defense Prostate Cancer Research Program DAMD, and NIH grant RO3 HD044783-01 to Dr Ozerdem, and by NIH grant RO1 CA95287 to Dr Stallcup.

References

1. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; 1: 27–31.
2. Risau W. Mechanisms of angiogenesis. *Nature* 1997; 386: 671–4.
3. Sims DE. Recent advances in pericyte biology – implications for health and disease. *Can J Cardiol* 1991; 7: 431–43.
4. Nehls V, Denzer K, Drenckhahn D. Pericyte involvement in capillary sprouting during angiogenesis *in situ*. *Cell Tissue Res* 1992; 270: 469–74.
5. Rhodin JA. Ultrastructure of mammalian venous capillaries, venules, and small collecting veins. *J Ultrastruct Res* 1968; 25: 452–500.
6. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003; 3: 401–10.
7. Darland DC, D'Amore PA. Blood vessel maturation: Vascular development comes of age. *J Clin Invest* 1999; 103: 157–8.
8. Sims DE. The pericyte – review. *Tissue Cell* 1986; 18: 153–74.
9. Ozerdem U, Grako KA, Dahlin-Huppe K et al. NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Dev Dyn* 2001; 222: 218–27.
10. Warren BA. The vascular morphology of tumors. In Peterson H-I (ed) *Tumor Blood Circulation: Angiogenesis, Vascular Morphology and Blood Flow of Experimental and Human Tumors*. Boca Raton, Florida: CRC Press 1979; 31–9.
11. Chang YS, di Tomaso E, McDonald DM et al. Mosaic blood vessels in tumors: Frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 2000; 97: 14608–13.
12. McDonald DM, Choyke PL. Imaging of angiogenesis: From microscope to clinic. *Nat Med* 2003; 9: 713–25.
13. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol* 2000; 156: 361–81.
14. Hendrix MJ, Sefter EA, Hess AR, Sefter RE. Angiogenesis: Vasculogenic mimicry and tumour-cell plasticity: Lessons from melanoma. *Nat Rev Cancer* 2003; 3: 411–21.
15. Sood AK, Sefter EA, Fletcher MS et al. Molecular determinants of ovarian cancer plasticity. *Am J Pathol* 2001; 158: 1279–88.
16. Ozerdem U, Monosov E, Stallcup WB. NG2 proteoglycan expression by pericytes in pathological microvasculature. *Microvasc Res* 2002; 63: 129–34.
17. O'Reilly MS, Holmgren L, Shing Y et al. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; 79: 315–28.
18. Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. *Cancer Res* 1975; 35: 218–24.
19. Kaighn ME, Narayan KS, Ohnuki Y et al. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 1979; 17: 16–23.
20. Horoszewicz JS, Leong SS, Chu TM et al. The LNCaP cell line – a new model for studies on human prostatic carcinoma. *Prog Clin Biol Res* 1980; 37: 115–32.
21. Ponten J, Macintyre EH. Long term culture of normal and neoplastic human glia. *Acta Pathol Microbiol Scand* 1968; 74: 465–86.
22. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 1992; 12: 954–61.
23. Greenberg NM, DeMayo F, Finegold MJ et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci USA* 1995; 92: 3439–43.
24. Ozaki H, Seo MS, Ozaki K et al. Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. *Am J Pathol* 2000; 156: 697–707.
25. Campochiaro PA. Retinal and choroidal neovascularization. *J Cell Physiol* 2000; 184: 301–10.
26. Grako KA, Ochiya T, Barritt D et al. PDGF (alpha)-receptor is unresponsive to PDGF-AA in aortic smooth muscle cells from the NG2 knockout mouse. *J Cell Sci* 1999; 112: 905–15.
27. Daniel CW, De Ome KB, Young JT et al. The *in vivo* life span of normal and preneoplastic mouse mammary glands: A serial transplantation study. *Proc Natl Acad Sci USA* 1968; 61: 53–60.
28. Gimbrone Jr, MA, Cotran RS, Leapman SB, Folkman J. Tumor growth and neovascularization: An experimental model using the rabbit cornea. *J Natl Cancer Inst* 1974; 52: 413–27.

29. Kenyon BM, Voest EE, Chen CC et al. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci* 1996; 37: 1625-32.
30. Drake CJ, Fleming PA. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* 2000; 95: 1671-9.
31. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res* 2003; 314: 15-23.
32. Dolbear F, Gratzner H, Pallavicini MG, Gray JW. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc Natl Acad Sci USA* 1983; 80: 5573-7.
33. Dean PN, Dolbear F, Gratzner H et al. Cell-cycle analysis using a monoclonal antibody to BrdUrd. *Cell Tissue Kinet* 1984; 17: 427-36.
34. Nowakowski RS, Lewin SB, Miller MW. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J Neurocytol* 1989; 18: 311-8.
35. Ezaki T, Baluk P, Thurston G et al. Time course of endothelial cell proliferation and microvascular remodeling in chronic inflammation. *Am J Pathol* 2001; 158: 2043-55.
36. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 1997; 277: 242-5.
37. Dawson B, Trapp RG. Basic and Clinical Biostatistics, 3rd ed. New York: McGraw-Hill 2001.
38. D'Amato R, Wszolowski E, Smith LE. Microscopic visualization of the retina by angiography with high-molecular-weight fluorescent-labeled dextrans in the mouse. *Microvasc Res* 1993; 46: 135-42.
39. Rhodin JA, Fujita H. Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. *J Submicrosc Cytol Pathol* 1989; 21: 1-34.
40. Sato Y, Rifkin DB. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-beta 1-like molecule by plasmin during coculture. *J Cell Biol* 1989; 109: 309-15.
41. Beck Jr, L, D'Amore PA. Vascular development: Cellular and molecular regulation. *FASEB J* 1997; 11: 365-73.
42. Hirschi KK, Rohovsky SA, Beck LH et al. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ Res* 1999; 84: 298-305.
43. Schlingemann RO, Rietveld FJ, de Waal RM et al. Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *Am J Pathol* 1990; 136: 1393-405.
44. Schlingemann RO, Rietveld FJ, Kwaspens F et al. Differential expression of markers for endothelial cells, pericytes, and basal lamina in the microvasculature of tumors and granulation tissue. *Am J Pathol* 1991; 138: 1335-47.
45. Schlingemann RO, Oosterwijk E, Wesseling P et al. Aminopeptidase a is a constituent of activated pericytes in angiogenesis. *J Pathol* 1996; 179: 436-42.
46. Wesseling P, Schlingemann RO, Rietveld FJ et al. Early and extensive contribution of pericytes/vascular smooth muscle cells to microvascular proliferation in glioblastoma multiforme: An immuno-light and immuno-electron microscopic study. *J Neuropathol Exp Neurol* 1995; 54: 304-10.
47. Morikawa S, Baluk P, Kaidoh T et al. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 2002; 160: 985-1000.
48. Gerhardt H, Golding M, Fruttiger M et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003; 161: 1163-77.
49. Hughes S, Chang-Ling T. Roles of endothelial cell migration and apoptosis in vascular remodeling during development of the central nervous system. *Microcirculation* 2000; 7: 317-33.
50. Le Lievre SA, Le Douarin NM. Mesenchymal derivatives of the neural crest: Analysis of chimeric quail and chick embryos. *J Embryol Exp Morphol* 1975; 34: 125-54.
51. Ait G, Lawrenson JG. Pericytes: Cell biology and pathology. *Cell Tissues Organs* 2001; 169: 1-10.
52. Nehls V, Drenckhahn D. Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J Cell Biol* 1991; 113: 147-54.
53. Balabanov R, Dore-Duffy P. Role of the CNS microvascular pericyte in the blood-brain barrier. *J Neurosci Res* 1998; 53: 637-44.
54. Boado RJ, Pardridge WM. Differential expression of alpha-actin mRNA and immunoreactive protein in brain microvascular pericytes and smooth muscle cells. *J Neurosci Res* 1994; 39: 430-5.
55. Alliot F, Rutin J, Leenen PJ, Pessac B. Pericytes and periendothelial cells of brain parenchyma vessels co-express aminopeptidase N, aminopeptidase A, and nestin. *J Neurosci Res* 1999; 58: 367-78.
56. Reuterdaal C, Sundberg C, Rubin K et al. Tissue localization of beta receptors for platelet-derived growth factor and platelet-derived growth factor B chain during wound repair in humans. *J Clin Invest* 1993; 91: 2065-75.
57. Sundberg C, Ljungstrom M, Lindmark G et al. Microvascular pericytes express platelet-derived growth factor-beta receptors in human healing wounds and colorectal adenocarcinoma. *Am J Pathol* 1993; 143: 1377-88.
58. Rajkumar VS, Sundberg C, Abraham DJ et al. Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. *Arthritis Rheum* 1999; 42: 930-41.
59. Grako KA, Stallcup WB. Participation of the NG2 proteoglycan in rat aortic smooth muscle cell responses to platelet-derived growth factor. *Exp Cell Res* 1995; 221: 231-40.
60. Chekenya M, Enger PO, Thorsen F et al. The glial precursor proteoglycan, NG2, is expressed on tumour neovasculature by vascular pericytes in human malignant brain tumours. *Neuropathol Appl Neurobiol* 2002; 28: 367-80.
61. Sims DE. Diversity within pericytes. *Clin Exp Pharmacol Physiol* 2000; 27: 842-6.
62. Abramsson A, Berlin O, Papayan H et al. Analysis of mural cell recruitment to tumor vessels. *Circulation* 2002; 105: 112-7.
63. Yang M, Baranov E, Wang JW et al. Direct external imaging of nascent cancer, tumor progression, angiogenesis, and metastasis on internal organs in the fluorescent orthotopic model. *Proc Natl Acad Sci USA* 2002; 99: 3824-9.
64. Bergers G, Song S, Meyer-Morse N et al. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 2003; 111: 1287-95.
65. Saharinen P, Alitalo K. Double target for tumor mass destruction. *J Clin Invest* 2003; 111: 1277-80.

Molecular regulation of the VEGF family

– inducers of angiogenesis and lymphangiogenesis

BRADLEY K. McCOLL, STEVEN A. STACKER and MARC G. ACHEN

Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria, Australia

McColl BK, Stacker SA, Achen MG. Molecular regulation of the VEGF family – inducers of angiogenesis and lymphangiogenesis. *APMIS* 2004;112:463–80.

The vascular endothelial growth factor (VEGF) family of secreted glycoproteins are critical inducers of angiogenesis (growth of blood vessels) and lymphangiogenesis (growth of lymphatic vessels). These proteins are attractive therapeutic targets for blocking growth of blood vessels and lymphatics in tumors and thereby inhibiting the growth and spread of cancer – in fact, the first VEGF inhibitor has recently entered the clinic for treatment of cancer. In addition, the VEGFs are being considered for stimulation of angiogenesis in the context of ischemic disease and lymphangiogenesis for treatment of lymphedema. These therapeutic possibilities have focused great interest on the molecular regulation of VEGF family members. Much has been learned in the past five years about the mechanisms controlling the action of the VEGFs, including the importance of hypoxia, proteolysis, transcription factors and RNA splicing. An understanding of these mechanisms offers broader opportunities to manipulate expression and activity of the VEGFs for treatment of disease.

Key words: VEGF-C; VEGF-D; lymphatics; proteolysis.

Marc G. Achen, Ludwig Institute for Cancer Research, PO Box 2008, Royal Melbourne Hospital, Victoria 3050, Australia. e-mail: Marc.achen@ludwig.edu.au

Angiogenesis and lymphangiogenesis – the development of new blood vessels and lymphatics from the pre-existing vasculature, respectively – are processes with integral roles in embryonic development and numerous diseases. Angiogenesis is the better understood of the two processes, in part due to the intense research focus placed upon the field because of the significance of blood vascular development for tumor growth and ischemic disease. Angiogenesis research has progressed to the point where the first molecular therapies targeting nascent blood vessels in cancer are reaching the clinic (1–4).

A lack of molecular markers specific to the lymphatic system has been an impediment to lymphangiogenesis research until recently, when

the identification of several such markers (reviewed in (5, 6)) has led to molecular insights into lymphangiogenesis. Numerous pathologies are associated with the lymphatics, such as the metastatic spread of cancer and lymphedema, and therapeutic strategies based upon the expanding body of lymphatic knowledge are now being considered (7–9).

The development of blood vessels and lymphatics depends upon interactions between the vascular endothelium and signalling molecules derived from the serum and extracellular matrix (ECM). Among these signalling molecules, the vascular endothelial growth factor (VEGFs) family of proteins is central to angiogenesis and lymphangiogenesis. The VEGFs are dimeric endothelial cell mitogens encoded by five genes in mammals: *VEGF* (also known as *VEGF-A*), *VEGF-B*, *VEGF-C*, *VEGF-D* and placenta growth factor (*PlGF*) (10). Members of the fam-

ily are related by the characteristic VEGF homology domain (VHD), containing receptor binding sites and a conserved cystine-knot motif (10, 11). Variations in mRNA splicing generate isoforms of several of the VEGFs, adding to the complexity of the family (12–21). Recent work has advanced the understanding of the functions of the VEGFs, and uncovered some of the mechanisms by which they are regulated. This review will discuss the molecular and physiological stimuli controlling the expression and activity of the VEGF family in healthy and diseased tissues.

THE BLOOD VASCULATURE

VEGFs in embryonic development and physiological angiogenesis

VEGF. Embryonic development of the blood vascular system commences with the process of vasculogenesis, whereby endothelial cell precursors differentiate to endothelial cells and associate to form the primary vascular plexus (22, 23). Subsequent angiogenesis gives rise to a more extensive vasculature (22, 23). In the healthy adult, angiogenesis is chiefly restricted to wound healing and the female reproductive system. Postnatal neo-vascularization has been shown to make use of endothelial precursor cells from the bone marrow, which are incorporated into the nascent vessels (24, 25). New vessels are therefore not exclusively derived from the cells of pre-existing vessels.

The essential role of VEGF for embryonic vasculogenesis has been established via gene disruption studies. Disruption of a single *VEGF* allele causes embryonic lethality by embryonic day (E) 11 as a result of defective vasculogenesis, angiogenesis and large vessel formation (26, 27). Postnatal ablation of *VEGF* causes death in mice up to approximately 4 weeks of age due to defects including impaired organ development and reduced vascularisation, probably stemming from reduced angiogenesis (28). Disruption of VEGF signalling is apparently not harmful after this stage, although corpus luteum angiogenesis in the adult is VEGF dependent (29). VEGF therefore appears to have a limited role in maintaining the mature vasculature, instead impacting on those systems with an ongoing dependence upon angiogenesis:

Examination of the postnatal vascularisation of the retina reveals the mechanism whereby new vessels are stabilised and made VEGF-independent. Newly formed vessels in the retina undergo regression in the absence of VEGF (30), as a result of endothelial cell apoptosis. Temporal studies of the developing retinal vasculature reveal that formation of the vascular plexus precedes recruitment of pericytes to the new vessels, and pericyte association is crucial for stabilisation of the neo-vasculature (31). Therefore VEGF is necessary to maintain immature vessels and, without full pericyte coverage, new vessels will regress in the absence of VEGF stimulation.

The effects of VEGF are mediated by binding to VEGFR-1 and VEGFR-2, receptor tyrosine kinases expressed on the blood vascular endothelium (Fig. 1). Both VEGFR-1 and VEGFR-2 are essential for embryonic vascular development and viability, although differences in the phenotypes of mice lacking the receptors reveal distinct functions. VEGFR-2 is believed to be the principal pro-angiogenic receptor, as deletion of the *VEGFR-2* gene results in failed vasculogenesis with a lack of organised vessels and haematopoietic precursors (32). VEGFR-1 ablation causes defective assembly of endothelial cells (33), related to the development of increased numbers of hemangioblasts (34). The VEGFR-1 intracellular tyrosine kinase domain is not essential for embryonic viability (35), sug-

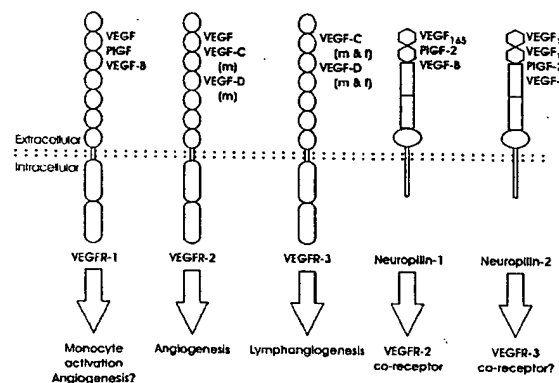


Fig. 1. Interaction of the VEGFs with receptors. Ligands for each receptor are listed. Where isoforms are not specified, all isoforms bind the receptor. Mature and full-length forms of VEGF-C and VEGF-D are denoted "m" and "f", respectively. The proposed function is listed below each receptor. The role of VEGFR-1 in angiogenesis is uncertain.

gesting that VEGFR-1 may be a negative regulator of VEGF activity.

Several isoforms of VEGF exist, generated by alternative mRNA splicing (12–16). The smallest isoform, VEGF₁₂₁ is freely soluble, whereas VEGF₁₈₉ and VEGF₂₀₆ are completely bound to the ECM (36). The predominant isoform, VEGF₁₆₅, exists as both soluble and ECM-bound forms. Emerging data show specific functions for the VEGF isoforms (37), possibly relating to the isoform-specific binding of VEGF to a second class of receptors, the Neuropilins. Neuropilin-1 (NRP-1) and Neuropilin-2 (NRP-2) are cell surface receptors associated with neuronal guidance (38). VEGF₁₆₅ binds both NRP-1 and NRP-2, but VEGF₁₄₅ binds NRP-2 only (39–41). Overexpression of the *NRP-1* gene is lethal at the embryonic stage, resulting in excessive capillary and blood vessel formation, thinning of the walls of the heart, and defects in other systems (42). Likewise, deletion of *NRP-1* causes death due to defects in embryonic vascularisation, although at later stages than those seen in VEGFR-2 deficient mice (43). *NRP-2* is not required for viability, but disruption of both *NRP-1* and *NRP-2* genes results in embryonic death due to severe vascular abnormalities, similar to those of VEGF or VEGFR-2 deficient mice (44). Furthermore, co-expression of NRP-1 with VEGFR-2 suggests that NRP-1 enhances VEGF binding to VEGFR-2 (40). NRP-1 may therefore function in concert with VEGFR-2 as a VEGF co-receptor during angiogenesis.

Despite the function of VEGF as a primary inducer of angiogenesis, some studies of VEGF-mediated initiation of blood vessel growth suggest that this growth factor alone is insufficient to establish mature vessels. Studies in which VEGF is elevated, in muscle from engineered myoblasts (45), transgenically in the skin (46) or systemically using adenoviral vectors (47) resulted in vascular leakage with associated inflammation. It seems that additional signalling molecules, most notably the Angiopoietins, their receptors the Ties, and the Ephrins are also necessary to give rise to fully functional vessels (23, 48–50).

VEGF-B. Although closely related to VEGF (19, 51), the physiological function of VEGF-B is less clear. Alternative RNA splicing generates

two isoforms of VEGF-B, VEGF-B₁₆₇ and VEGF-B₁₈₆ (20), the larger of which is the predominant mRNA species (52). Both isoforms are ligands for VEGFR-1 and NRP-1 (Fig. 1), although VEGF-B₁₈₆ requires proteolytic cleavage of the C-terminal region for binding to NRP-1 (53) (Fig. 2). Neither isoform binds VEGFR-2 or VEGFR-3 (53, 54). VEGF-B induces a mitogenic response in endothelial cells *in vitro* (51), and expression of VEGF-B in the embryonic heart and adult cardiac and skeletal muscle suggests a role in vascularisation of the musculature (51). However, VEGF-B does not appear to be strongly angiogenic as indicated by adenoviral delivery to periadventitial tissue (55) or hindlimb skeletal muscle (56). Unlike VEGF, mice lacking VEGF-B are viable, but suffer from smaller hearts, abnormal coronary vasculature and defective recovery from cardiac ischemia (57). Certain VEGF isoforms are also required for development of the heart (37), and coexpression studies have shown that VEGF and VEGF-B can form heterodimers (51). Together these data indicate a role for VEGF-B in cardiac development, possibly in cooperation with VEGF.

PlGF. PlGF is expressed in the placenta throughout pregnancy, and also in the heart, lung, brain and skeletal muscle (58, 59). Alternative splicing of the human primary transcript generates three isoforms: PlGF₁₃₁ (PlGF-1), PlGF₁₅₂ (PlGF-2) and PlGF₂₀₃ (PlGF-3) (17, 18). Like VEGF-B, PlGF is a ligand for VEGFR-1 but not VEGFR-2 (58, 60), and PlGF-2 is able to bind NRP-1 and NRP-2 (61, 62) (Fig. 1). PlGF homodimers are poor inducers of angiogenesis *in vivo*, and only provoke a weak mitogenic response from endothelial cells (60, 63). VEGF can form heterodimers

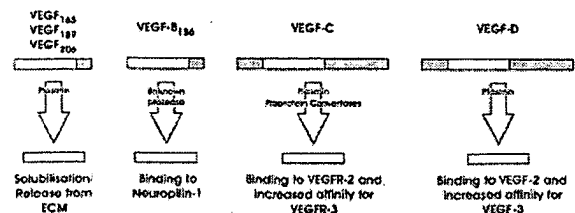


Fig. 2. Proteolytic activation of the VEGFs. Shading represents regions of the protein removed by proteolysis. Functional outcomes of proteolysis are listed below each growth factor.

with PlGF(63), although conflicting evidence exists as to the capacity of such heterodimers to induce angiogenesis (63, 64). Deletion of the *PlGF* gene in mice does not cause significant defects in vascular development (65), although overexpression of *PlGF* in the skin results in increased vessel formation and permeability (66), demonstrating a pro-angiogenic role for the growth factor. PlGF stimulates angiogenesis as well as migration and proliferation of endothelial cells *in vitro* in a manner dependent upon VEGF and VEGFR-1 (65). A model has been suggested to explain these observations (65), in which PlGF binding to VEGFR-1 signals for angiogenesis, whereas VEGF binding does not. PlGF binding to VEGFR-1 would displace VEGF from the receptor, increasing the amount of soluble VEGF available for pro-angiogenic binding to VEGFR-2. The VEGFR-1 tyrosine kinase domain is not essential for embryonic vascular development (35), indicating that VEGFR-1 acts as a non-signalling receptor which is consistent with the normal vascular development observed in PlGF deficient embryos (65).

In vivo studies of ischemia have shown that PlGF treatment stimulates arteriogenesis, the enlargement of pre-existing arterioles (67, 68). Ablation of the *PlGF* gene inhibits this process, a phenotype which can be rescued by bone marrow transplantation (69). PlGF is capable of stimulating migration and tissue factor production in monocytes (70), and PlGF treatment results in elevated extravasation of macrophages (68). These lines of evidence concur with the observed contribution of bone marrow derived endothelial cell precursors to vessel formation (25), indicating a role for haematopoietic cells in angiogenesis, in response to stimulation by PlGF.

Pathological angiogenesis

Aberrant or insufficient vascularisation of tissues is a feature of several pathologies, including arthritis, atherosclerosis, diabetic retinopathy and tumor growth. In the case of cancer, access to the blood supply and the nutrients therein is a necessary precursor for significant tumor growth, making tumor angiogenesis a target for emerging therapeutics (71–74). In contrast to developmental angiogenesis, tumor angiogenesis results in irregularly shaped, convoluted vessels that exhibit disorganised or even

reversed blood flow (73). Furthermore, tumor derived vasculature is often leaky, a characteristic of immature blood vessels (75). Tumor angiogenesis may incorporate cancer cells into the wall of the developing vessel (76), and tumor models have been reported in which vessel growth involves the recruitment of circulating endothelial precursor cells, suggesting that new vessels are not solely derived from pre-existing vasculature (25). It has also been hypothesised that some tumor cell types may form vessel-like structures independently of the endothelium (77). Tumor angiogenesis therefore appears to be a somewhat chaotic process, in which normal signalling molecules are co-opted in a disorganised manner to provide the tumor with improved access to the blood supply (73).

The evidence for VEGF as a primary inducer of tumor angiogenesis is extensive. Animal xenograft models of cancer in which the *VEGF* gene has been disrupted, or VEGF signalling blocked, show drastically reduced growth of tumors, demonstrating the significance of VEGF in tumor growth (27, 78–80). Surveys of human cancers reveal significant elevation of VEGF expression in the majority of tumors examined, including those from the lung, breast, gastrointestinal tract, ovary and colon (81–85). Expression of VEGF correlates with increased tumor vascularisation and poor patient prognosis (81, 86, 87), suggesting VEGF as a therapeutic target, and leading to the development of therapies based upon inhibition of VEGF activity. The approval of the US Food and Drug Administration of Avastin™, a neutralizing antineutralizing antibody to VEGF, for treatment of metastatic colorectal cancer has focussed great interest on the field of anti-angiogenesis as an approach to cancer therapy (1, 4).

PlGF may be associated with pathological angiogenesis as demonstrated by decreased vascularisation of tumor models lacking PlGF (65), although expression of PlGF has been reported to be variable in human cancers (88–90). Inhibition of the PlGF receptor VEGFR-1 suggests a role for PlGF in tumor angiogenesis as well as arthritis and atherosclerosis (67), possibly through synergistic effects with VEGF (reviewed in (91)). Examination of VEGF-B in tumors reveals little conclusive data to indicate a substantial role for VEGF-B in tumor progression (92, 93), although isoform specific vari-

ation of VEGF-B expression in tumors has been reported (52).

Molecular regulation of angiogenic VEGFs *VEGF*

Hypoxia. Cellular growth and proliferation beyond the dimensions through which oxygen can readily diffuse results in reduced oxygen tension in the tissue. Within the cell, a number of metabolic alterations occur in response to hypoxia, including the synthesis of pro-angiogenic growth factors targeting the vascular endothelium, most notably VEGF (94). Hypoxia has been demonstrated to be a major regulator of VEGF both *in vitro* and *in vivo* (95–97) (Table 1), and VEGF mRNA is elevated in ischemic tumor cells adjacent to areas of necrosis (98). These observations have led to efforts to identify inhibitors of the hypoxic response as novel cancer therapeutics (99).

The cellular response to hypoxia is mediated by the hypoxia-inducible transcription factor (HIF-1), a heterodimeric protein which binds to hypoxia response elements (HRE) in the promoter/regulatory regions of hypoxia-inducible genes, including the *VEGF* gene, and initiates transcription by recruitment of transcriptional activators such as CREB/p300 (100). Under normoxic conditions, the α subunit of HIF-1 is degraded via the ubiquitin-proteasome pathway, preventing formation of active HIF-1 (for review of HIF-1 regulation see (101)). The ubiquitination step is mediated by the von Hippel-Lindau (VHL) protein (102), which recognises hydroxylated proline residues in HIF-1 α (101). Proline hydroxylation of HIF-1 α is cata-

lysed by a family of three prolyl hydroxylases utilising molecular oxygen as a substrate (103). Studies of recombinant prolyl hydroxylases demonstrate that the affinity of these enzymes for oxygen is such that their activity is reduced at oxygen concentrations observed in hypoxic tissues (104), leading to reduced ubiquitination-degradation of HIF-1 α . Hydroxylation of an asparagine residue also regulates the activity of the C-terminal transactivation domain of HIF-1 α by blocking interactions with the transcriptional activator p300 (105). As with the prolyl hydroxylases, activity of the enzyme responsible is dependent upon oxygen concentration at physiological levels (106, 107). Hydroxylation of HIF-1 α therefore acts as a hypoxia sensitive switch linking local oxygen concentrations to the stability and activity of the transcription factor, and hence to VEGF expression.

Genes associated with transformation. Numerous genes associated with tumorigenic transformation are also involved with regulation of VEGF expression (Table 1). Examination of the *VEGF* gene promoter region reveals the presence of consensus AP-1 sequences, sites recognised by the c-fos family of transcription factors (15). The c-fos family are proto-oncogenes which activate transcription by binding to AP-1 sites in transcriptional promoters (for review see (108)). Since fos is upregulated by treatment with phorbol esters and various growth factors, VEGF expression could also be elevated in response to these stimuli, as is indeed the case (15, 109–112).

The *ras* oncogene is another logical candidate to activate VEGF expression, as *ras* activity is a

TABLE 1. *Regulators of the VEGFs. Arrows indicate positive (\uparrow) or negative (\downarrow) stimuli*

VEGF	PIGF	VEGF-B	VEGF-C	VEGF-D
Regulation of transcription				
\uparrow Hypoxia				
\uparrow Inflammatory mediators/COX-2	\uparrow Hypoxia \uparrow FoxD1		\uparrow Inflammatory mediators/COX-2	\uparrow AP-1 transcription factors
\uparrow AP-1 transcription factors	transcription factor			\uparrow Cell-cell contact
\uparrow Mutant <i>ras</i>				
\uparrow Mutant p53				
Post-transcriptional regulation				
\uparrow Proteolysis (release from ECM)		\uparrow Proteolysis	\uparrow Proteolysis	\uparrow Proteolysis \downarrow Cytosolic β -catenin

component of the signal transduction pathway linking extracellular stimuli to AP-1 mediated activation of transcription (108). Oncogenic, activated ras stimulates VEGF expression in cell lines (113, 114), and ras activity is associated with VEGF expression, tumorigenicity and angiogenesis in mouse models of cancer (115, 116). Therefore oncogenic ras may play a role in stimulating angiogenesis as well as driving proliferation of tumor cells in cancer.

A transcriptionally inactive mutant of the tumor suppressor p53 causes elevated VEGF expression (117), and wild-type p53 represses VEGF transcription (118). Deletion of the p53 gene in tumor cells results in enhanced vascularisation and tumor growth, and p53-deficient cells show increased induction of VEGF under hypoxic conditions (119). The p53 protein promotes ubiquitination and degradation of HIF-1 α (119), hence VEGF expression is enhanced by HIF-1 in the absence of p53. In addition to upregulating VEGF, mutation of p53 may confer resistance to antiangiogenic therapies, as hypoxia-induced apoptosis is p53 dependent. A p53 mutant tumor model has shown reduced responsiveness to therapies targeting the vasculature (120), demonstrating that p53 status may be a factor when considering anti-VEGF therapies.

RNA splicing isoforms and proteolysis. VEGF exists as a number of isoforms derived from mRNA splice variants (12, 14–16). The isoforms differ at the C terminus, with the larger variants exhibiting substantial affinity for heparin (36). The smallest isoform, VEGF₁₂₁, does not bind heparin and is freely soluble upon secretion, whereas the higher molecular weight isoforms bind to the cell surface/extracellular matrix (ECM) (36, 121). VEGF isoforms sequestered in this way can be released from the ECM by the action of heparinase or the fibrinolytic serine protease plasmin, giving rise to soluble, biologically active molecules (36, 121) (Fig. 2). It has been proposed that the ECM-bound forms of VEGF represent a pool of available growth factor, which can be activated in the course of tissue remodelling in conjunction with the degradation of ECM components necessary for neovascularisation (121).

Inflammatory cytokines and cyclooxygenase-2 (COX-2). The inflammatory condition rheu-

matoid arthritis is characterised by proliferation of the synovium, including large numbers of blood vessels, and invasion of the adjacent cartilage. Angiogenesis is a significant component of this process, believed to be driven by local production of angiogenic growth factors (91). Inflammatory mediators such as IL-1 α , IL-1 β , TGF- β and prostaglandin E2 (PGE2) induce VEGF expression in a number of cell types, including umbilical vein endothelial cells, smooth muscle cells and synovial fibroblasts (111, 122–125). VEGF is therefore a potential target for intervention in inflammatory disease. Furthermore, the enzyme COX-2 is an inducible component of the inflammatory prostaglandin synthesis pathway, and is upregulated in many human cancers (126). Inhibitors of the enzyme demonstrate anti-angiogenic activity in animal models of cancer, suggesting that COX-2 activity may be a target for therapies acting indirectly on VEGF (127, 128).

PlGF and VEGF-B

Like VEGF, PlGF is also induced by hypoxia (129), and recent work has shown *PlGF* expression to be activated by FoxD1, a member of the Forkhead/Winged Helix family of transcription factors associated with branching of the ureteric bud in the kidney (130). *PlGF*-null mice have not been reported to develop renal defects, indicating either a non-essential or redundant role for PlGF in the kidney. In addition, PlGF is upregulated in keratinocytes during wound healing (65, 131). The three human isoforms of PlGF differ in their affinities for the ECM, with PlGF-2 incorporating a sequence of 21 amino acids necessary for binding to heparin (17, 132). VEGF and PlGF are therefore both regulated according to tissue oxygenation and matrix interactions, demonstrating the interplay between vascular development and the ECM.

VEGF-B does not respond to hypoxia, Ras oncoprotein or several other stimuli known to regulate other VEGF family members (112, 133, 134), and the mechanisms whereby *VEGF-B* gene expression is regulated remain unclear.

THE LYMPHATIC VASCULATURE

The lymphatic vasculature is composed of a network of blind-ended, thin-walled vessels and

capillaries lined by a continuous layer of endothelial cells (135). Extravasated fluid and solutes from the extracellular spaces are collected by the initial lymphatics, then passed through a network of progressively larger lymphatic vessels, finally returning to the blood circulation via the thoracic duct (136). In addition, the lymphatic system is a major site of immune surveillance as stimulated dendritic cells migrate to the lymph nodes where foreign antigens are presented to lymphocytes (137).

Embryonic development and physiological lymphangiogenesis

The lymphatic vessels develop subsequent to the formation of the blood vasculature, leading to the suggestion that they are derived from the blood vessels (138). Recent advances in the understanding of lymphatic development have shown that expression of the homeobox transcription factor Prox-1 by a population of endothelial cells in the cardinal vein during embryogenesis is a defining characteristic of differentiation to the lymphatic phenotype (139, 140). Subsequent budding and sprouting of the Prox-1 positive cells gives rise to the lymphatic vasculature (for review see (141)).

Two members of the VEGF family, VEGF-C and VEGF-D, have been shown to act as lymphangiogenic growth factors (142, 143). Expression of either growth factor in the skin of transgenic mice results in lymphatic hyperplasia without altering the blood vasculature (143, 144). Disruption of the *VEGF-C* gene demonstrates that the growth factor is indispensable for embryonic lymphangiogenesis (145). Embryos carrying a homozygous deletion of *VEGF-C* are not viable, and fail to form the initial lymph sacs which generate the lymphatic vasculature. Prox-1 expression was detected in endothelial cells of VEGF-C deficient embryos, but the Prox-1 positive cells failed to migrate from the cardinal vein (145), indicating that VEGF-C is required for migration of the endothelial cells which go on to form the lymphatic system. *VEGF-C* exhibits a gene dosage effect as mice carrying only one functional *VEGF-C* allele are prone to lymphedema (145). The role of VEGF-D during embryonic development is unknown.

In the human, VEGF-C and VEGF-D are ligands for VEGFR-2 and the related receptor

tyrosine kinase VEGFR-3 (146, 147), although mouse VEGF-D binds only VEGFR-3 (148). In the early embryo VEGFR-3 is broadly expressed on the endothelium, but in time becomes restricted to the lymphatic endothelium (149), leading to the suggestion that VEGFR-3 signals for lymphangiogenesis. Functional studies support this hypothesis, demonstrating that VEGFR-3 transduces mitogenic and migratory signals in lymphatic endothelial cells, and stimulation of the receptor blocks apoptosis induced by serum starvation (150). Furthermore, expression in the skin of a mutant form of VEGF-C specific for VEGFR-3 causes lymphatic hyperplasia without altering blood vessel structure (143), and disruption of VEGFR-3 signalling in the same model blocks development of the lymphatic phenotype (143). VEGFR-3 is therefore a major transducer of lymphangiogenic signalling in the adult. In addition, deletion of the *VEGFR-3* gene results in embryonic death due to defective remodelling of the large blood vessels (151). VEGFR-3 therefore has a role in embryonic vascular development prior to the emergence of the lymphatics.

VEGF-C has also been shown to bind to NRP-2 (152), and deletion of the *NRP-2* gene impairs formation of the small lymphatics (153), leading to the hypothesis that NRP-2 may act as a co-receptor for VEGFR-3, in a fashion analogous to NRP-1 acting as a co-receptor for VEGFR-2 (Fig. 1).

Examination of VEGF-C and VEGF-D function in a number of assays has also shown an angiogenic activity for the growth factors (154–158), presumably via activation of VEGFR-2. Gene delivery experiments using adenoviruses expressing VEGFs show induction of angiogenesis by VEGF-C and VEGF-D *in vivo* (55, 56). Although the physiological significance of VEGF-C and VEGF-D in angiogenesis is unclear the promising results achieved so far with gene delivery approaches suggest a role for the factors in therapy for ischemic disease.

Pathological lymphangiogenesis and lymphatic dysfunction

Cancer. Given the propensity for numerous types of cancer to metastasize via the lymphatic system, it has been proposed that tumors may stimulate lymphangiogenesis in a manner analogous to tumor angiogenesis, thereby promot-

ing lymphogenous metastasis (6, 159). The production of lymphangiogenic growth factors is believed to stimulate lymphatic vessel development in the region of the tumor, enabling cancer cells to gain access to the lymphatic vasculature.

A number of animal models have been utilised to examine the potential of VEGF-C and VEGF-D to promote lymphatic metastasis. Studies in which VEGF-C or VEGF-D were expressed in transplanted tumor cells or transgenic tumor models have demonstrated that these growth factors promote tumor lymphangiogenesis and lymphatic metastasis (160–164). Furthermore, clinicopathological studies of these lymphangiogenic growth factors in cancer reveal that, in many instances, expression of VEGF-C or VEGF-D does indeed correlate with the capacity of a tumor to metastasize (165–170). Analysis of xenograft tumor models has revealed that VEGF-C and VEGF-D can also drive tumor angiogenesis and accelerate solid tumor growth (158, 164), potentially as a result of VEGFR-2 activation. For a complete discussion of VEGF-C and VEGF-D and tumor lymphangiogenesis, see Stacker *et al.*, this issue.

Lymphedema. A spectrum of pathologies exists stemming from inadequate lymphatic function (for review of disorders of the lymphatic system see (135, 171)). Although sharing the common feature of fluid accumulation in tissues as a result of inadequate lymphatic drainage (lymphedema), the underlying causes may be either familial or acquired. Specific genetic lesions have been identified in a few instances of inherited lymphedema, providing insights into lymphangiogenesis. Specifically, mutations in the *VEGFR-3* gene have been identified in a number of cases (172, 173), and the forkhead transcription factor *FOXC2* is also implicated in congenital lymphedema (174). Acquired lymphedema can be the result of infection (commonly filariasis) or lymph node resection and radiation treatment of cancer. Regardless of the cause, current treatments for lymphedema are targeted towards alleviating symptoms rather than treating the underlying causes (171). A better understanding of the molecular control of lymphangiogenesis offers the potential to develop novel treatments for this painful and disfiguring condition based on stimulating lymphatic

development to enhance or restore lymphatic function (7, 8).

Molecular regulation of lymphangiogenic VEGFs

Proteolytic activation of VEGF-C and VEGF-D. VEGF-C and VEGF-D are both synthesised as proproteins, with the central receptor binding VEGF homology domain (VHD) flanked by N- and C-terminal propeptides (146, 147). During biosynthesis the propeptides are cleaved off, yielding the mature VHD (175, 176). As a consequence of proteolysis VEGF-C acquires the capacity to bind to VEGFR-2 (175) and a recombinant mature form of VEGF-D shows an approximately 290-fold greater affinity for VEGFR-2 than unprocessed VEGF-D (176). Full-length forms of both growth factors bind to VEGFR-3, but do so with greater affinity after proteolytic maturation (175, 176). In support of these observations, mutation of one of the sites of proteolytic cleavage in VEGF-C has been shown to reduce lymphangiogenesis and angiogenesis induced by VEGF-C in a mouse tumor model (177).

The capacity of the mature forms of VEGF-C and VEGF-D to act as ligands for VEGFR-2 explains how these proteins stimulate angiogenesis in addition to lymphangiogenesis (156–158, 164). The proteolysis-dependent binding to VEGFR-2 may represent a mechanism whereby the angiogenic activity of VEGF-C and VEGF-D is activated.

The marked effect of the proteolytic activation of VEGF-C and VEGF-D on their affinity for VEGFR-2 and VEGFR-3 indicates that the enzymes carrying out this processing are important regulators of lymphangiogenesis and angiogenesis. Treatment of VEGF-C and VEGF-D with the fibrinolytic serine protease plasmin was recently shown to generate fully processed, mature forms of the VHD with greatly enhanced capacities to activate both VEGFR-2 and VEGFR-3 (178). Since plasmin can also release ECM-bound VEGF, this enzyme is capable of activating both angiogenic (36) and lymphangiogenic growth factors (Fig. 2). These findings suggest that plasmin is a master regulatory molecule that co-ordinates lymphangiogenesis, angiogenesis and fibrinolysis during wound healing.

Some members of the furin/proprotein con-

vertase (PC) family of proteases have also been shown to partially activate VEGF-C (177). The PCs are a family of ubiquitously expressed proteases capable of activating a range of precursor proteins by cleavage downstream of dibasic residues. Substrates for the PCs include numerous growth factors, zymogens, receptors and viral proteins (for review of PC function see (179, 180)). The PCs furin, PC5 and PC7 are able to cleave VEGF-C at the C-terminal site of proteolysis, thereby cleaving the C-terminal propeptide from the VHD (177). However, it is not known if PCs cleave the N-terminal propeptide from the VHD, nor if they process VEGF-D.

These observations suggest a model in which VEGF-C and VEGF-D may be activated in various biological contexts. Firstly, regulated expression of PCs could activate VEGF-C and VEGF-D during embryogenesis, when plasmin is unlikely to play a role (note that mice deficient for plasminogen, the plasmin precursor, are viable and have not been reported to suffer lymphedema (181)). Plasmin could activate VEGF-C and VEGF-D from a pool of inactive full-length molecules in response to tissue damage in adults. It is known that both VEGF-C and VEGF-D are localised in vascular smooth muscle in adult human tissues (182, 183). Local activation of these molecules in response to tissue damage and plasmin production could represent a mechanism for stimulating rapid vessel repair during wound healing. In addition, activation of VEGF-C and VEGF-D by plasmin may be associated with tumor lymphangiogenesis, given that plasminogen deficient mice show delayed formation of lymph node metastases when inoculated with Lewis lung carcinoma cells (184). Upregulation of VEGFR-2 and VEGFR-3 on blood vessels during wound healing and tumor angiogenesis (185) may facilitate the development of new vessels.

Induction of VEGF-C expression by inflammatory cytokines. Interleukin-1 (IL-1) and Tumor Necrosis Factor- α (TNF- α) stimulate VEGF-C expression in human lung fibroblasts and human umbilical vein endothelial cells (HUVEC) (186). In conjunction with inflammation-induced VEGF expression and angiogenesis, expression of VEGF-C and consequent lymphangiogenesis may have a role in maintaining fluid balance in the inflamed tissue. Also, an immune

response at the site of inflammation may depend upon increased lymphatic function in the affected area. It therefore seems logical that inflammatory mediators can induce expression of a lymphangiogenic growth factor such as VEGF-C.

VEGF-C and COX-2. The association of VEGF-C with the inflammatory response extends to COX-2. Recent work has shown COX-2 to be capable of upregulating VEGF-C expression in human lung adenocarcinoma cells (187). In addition to VEGF-C, COX-2 activity and some prostaglandins produced by COX-2 (188) also elevate angiopoietin-2, another protein required for lymphangiogenesis (189). COX-2 is therefore an inducer of two proteins integral to lymphangiogenesis. This relationship has significant implications for tumor lymphangiogenesis and metastasis, given the earlier observation that elevated COX-2 expression may be associated with lymph node metastasis from adenocarcinoma of the lung (190). COX-2 may therefore be a major inducer of angiogenic and lymphangiogenic proteins, and a potential target for anti-metastatic therapies based upon existing anti-inflammatory compounds.

VEGF-D induction by fos. The mouse homologue of VEGF-D was originally identified as a novel c-fos responsive gene (191), and subsequent investigation of the X-linked human VEGF-D gene (192) revealed a canonical AP-1 binding site in the transcriptional promoter (192, 193). It has been reported that VEGF-D levels are high in glioblastoma multiforme (GBM), yet levels of c-fos are very low in this tumor (194). However, the fos-related antigen-1 (fra-1), another AP-1 transcription factor, is elevated in GBM and induces VEGF-D expression (194). Therefore, the AP-1 transcriptional activation pathway appears to be a regulator of VEGF-D expression. Although the significance of this relationship in healthy physiology is unclear, the association between increased transcription mediated by AP-1 and tumorigenesis is well established (108). Therefore, elevated AP-1 activity may lead not only to neoplasia, but also to tumor angiogenesis and lymphangiogenesis via elevated VEGF-D expression.

VEGF-D and cell-cell contact. The cadherins are a family of calcium-dependent cell surface pro-

teins which mediate cell-cell adhesion through homotypic binding with their counterparts on adjacent cells (195). VEGF-D expression has been shown to be enhanced by cadherin-11 *in vitro* whereas inhibition of cadherin-11 expression reduces VEGF-D induction (196). In addition, VEGF-D mRNA stability is downregulated by cytosolic β -catenin (197). β -catenin exists either as a structural protein at the cell membrane in a complex with the cadherins, or in the cytoplasm. Repression by cytosolic β -catenin and upregulation in response to cadherin-11 implies that VEGF-D expression is dependent upon stable cellular interactions with the surrounding environment, and may have implications for the secretion of lymphangiogenic growth factors by migrating cells.

CONCLUDING REMARKS

Numerous members of the VEGF family share common mechanisms of regulation. Regulating the angiogenic and lymphangiogenic activities of the VEGFs via common pathways may allow coordinated development of the lymphatic and blood vasculature, necessary for fluid homeostasis.

The existence of multiple protein isoforms generated by variations in RNA splicing has been reported for all mammalian VEGFs except VEGF-C. In the case of VEGF (14–16), VEGF-B (19, 20) and PlGF (17, 18) the generation of isoforms with different affinities for heparin and the ECM/cell surface regulates the biological distribution of these molecules. However, the significance of the two isoforms of mouse VEGF-D (21), that differ in the C-terminal propeptide, is as yet unknown.

Proteolysis regulates several VEGFs (Fig. 2), and significantly influences receptor affinity of one isoform of VEGF-B (53), and receptor affinity and specificity of VEGF-C (175) and VEGF-D (176). Proteolysis also modulates VEGF activity, by releasing ECM-bound forms of VEGF (36). The findings that plasmin can activate both VEGF-C and VEGF-D (178), in addition to releasing VEGF from the ECM (36), indicate the potential importance of this protease for co-ordinating angiogenesis, lymphangiogenesis and fibrinolysis in wound healing.

Hypoxia is a critical stimulus for the angio-

genic factors VEGF (95–98) and PlGF (129); however, there is no compelling evidence that it drives the expression of the lymphangiogenic growth factors VEGF-C and VEGF-D. This seems logical given that modulation of tissue oxygenation is not a direct function of the lymphatic vasculature.

The identification of the VEGFs as inducers of angiogenesis and lymphangiogenesis has led to new therapeutic possibilities for diseases associated with vascular function. These include stimulation of these processes using recombinant proteins or gene delivery systems to treat ischemic disease and lymphedema. Additionally, pathological angiogenesis and lymphangiogenesis in solid tumors could be blocked by inhibitors of these growth factors, such as monoclonal antibodies and soluble receptors. However, as our understanding of the mechanisms regulating the VEGFs becomes more extensive there will be opportunities to manipulate the stimuli that influence the production or activity of these growth factors. For example, it may prove clinically attractive to inhibit the proteases that activate the lymphangiogenic growth factors in order to block formation of tumor lymphatics and the resultant lymphogenous metastasis. Likewise, inhibitors of the HIF-1 hypoxia response pathway may be of benefit to block production of VEGF and resultant tumor angiogenesis (99). Future advances in our understanding of the biological regulation of the VEGFs will reveal more potential approaches for manipulating angiogenesis and lymphangiogenesis in the clinic.

This work was supported by the National Health and Medical Research Council of Australia (NH&MRC). BKM is supported by a Melbourne Research Scholarship from the University of Melbourne, SAS by a Senior Research Fellowship from the Pharmacia Foundation, and MGA by a Senior Research Fellowship from the NH&MRC. We thank Tony Burgess for helpful comments.

REFERENCES

1. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–34.

2. Fiedler W, Mesters R, Tinnefeld H, Loges S, Staib P, Duhrsen U, et al. A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. *Blood* 2004;102:2763-67.
3. Willett CG, Boucher Y, di Tomaso E, Duda EG, Munn LL, Tong RT et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivasculature effects in human rectal cancer. *Nat Med* 2004;10:145-7.
4. Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 2003;21:60-5.
5. Stacker SA, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J* 2002;16:922-34.
6. Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K. Lymphangiogenesis and cancer metastasis. *Nat Rev Cancer* 2002;2:573-83.
7. Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, et al. Therapeutic lymphangiogenesis with human recombinant VEGF-C. *FASEB J* 2002;16:1985-7.
8. Saaristo A, Veikkola T, Tammela T, Enholm B, Karkkainen MJ, Pajusola K, et al. Lymphangiogenic gene therapy with minimal blood vascular side effects. *J Exp Med* 2002;196:719-30.
9. Yoon Y, Murayama T, Graveriaux E, Tkebuchava T, Silver M, Curry C et al. VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. *J Clin Invest* 2003;111:717-25.
10. Achen MG, Stacker SA. The vascular endothelial growth factor family; proteins which guide the development of the vasculature. *Int J Exp Pathol* 1998;79:255-65.
11. McDonald NQ, Hendrickson WA. A structural superfamily of growth factors containing a cystine knot motif. *Cell* 1993;73:421-4.
12. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246:1306-9.
13. Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J, Lau K, et al. Vascular endothelial growth factor: a new member of the platelet-derived growth factor gene family. *Biochem Biophys Res Commun* 1989;165:1198-206.
14. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991;5:1806-14.
15. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991;266:11947-54.
16. Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, et al. VEGF₁₄₅, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. *J Biol Chem* 1997;272:7151-8.
17. Maglione D, Guerriero V, Viglietto G, Ferraro M, Aprelikova A, Alitalo K, et al. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene* 1993;8:925-31.
18. Cao Y, Ji WR, Qi P, Rosin A, Cao YY. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochem Biophys Res Commun* 1997;235:493-8.
19. Grimmond S, Lagercrantz J, Drinkwater C, Silins G, Townson S, Pollock P, et al. Cloning and characterization of a novel human gene related to vascular endothelial growth factor. *Genome Res* 1996;6:124-31.
20. Olofsson B, Pajusola K, von Euler G, Chilov D, Alitalo K, Eriksson U. Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. *J Biol Chem* 1996;271:19310-17.
21. Baldwin ME, Roufail S, Halford MM, Alitalo K, Stacker SA, Achen MG. Multiple forms of mouse vascular endothelial growth factor-D are generated by RNA splicing and proteolysis. *J Biol Chem* 2001;276:44307-14.
22. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386:671-4.
23. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000;407:242-8.
24. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999;85:221-8.
25. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;7:1194-201.
26. Carmeliet P, Ferreira V, Breier G, Pollofeyt S, Kieckens L, Gertenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435-9.
27. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380:439-43.

28. Gerber H-P, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangel L *et al.* VEGF is required for growth and survival in neonatal mice. *Development* 1999;126:1149-59.
29. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, *et al.* Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 1998;4:336-40.
30. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995;1:1024-8.
31. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 1998;125:1591-8.
32. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, *et al.* Failure of blood-island formation and vasculogenesis in *flt-1* deficient mice. *Nature* 1995;376:62-6.
33. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the *Flt-1* receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995;376:66-70.
34. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in *flt-1* knock-out mice. *Development* 1999;126:3015-25.
35. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. *Flt-1* lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci USA* 1998;95:9349-54.
36. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992;267:26031-7.
37. Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, *et al.* Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF₁₆₄ and VEGF₁₈₈. *Nat Med* 1999;5:495-502.
38. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, Herzog Y. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovasc Med* 2002;12:13-9.
39. Soker S, Fidler IJ, Neufeld G, Klagsbrun M. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumour cells that bind VEGF₁₆₅ via its exon 7-encoded domain. *J Biol Chem* 1996;271:5761-67.
40. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;92:735-45.
41. Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 and neuropilin-1 are receptors for the 165-amino acid form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145- amino acid form of VEGF. *J Biol Chem* 2000;275:18040-5.
42. Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H. Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 1995;121:4309-18.
43. Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, *et al.* A requirement for neuropilin-1 in embryonic vessel formation. *Development* 1999;126:4895-902.
44. Takashima S, Kitakaze M, Asakura M, Asanuma H, Sanada S, Tashiro F, *et al.* Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc Natl Acad Sci USA* 2002;99:3657-62.
45. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell* 1998;2:549-58.
46. Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, *et al.* Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J Invest Dermatol* 1998;111:1-6.
47. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, *et al.* Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000;6:460-3.
48. Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, *et al.* Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 1999;13:295-306.
49. Lobov IB, Brooks PC, Lang RA. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival *in vivo*. *Proc Natl Acad Sci USA* 2002;99:11205-10.
50. Thurston G. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. *Cell Tissue Res* 2003;314:61-8.
51. Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, *et al.* Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996;93:2576-81.
52. Li X, Aase K, Li H, von Euler G, Eriksson U. Isoform-specific expression of VEGF-B in nor-

- mal tissues and tumors. *Growth Factors* 2001; 19:49–59.
53. Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, et al. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J Biol Chem* 1999;274:21217–22.
 54. Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, et al. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proc Natl Acad Sci USA* 1998;95:11709–14.
 55. Bhardwaj S, Roy H, Gruchala M, Viita H, Kholova I, Kokina I, et al. Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. *Hum Gene Ther* 2003;14:1451–562.
 56. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, et al. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res* 2003;92:1098–106.
 57. Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M, et al. Mice lacking the vascular endothelial growth factor-B gene (*Vegfb*) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res* 2000;86:E29–E35.
 58. Maglione D, Guerriero V, Viglietto G, Delli Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA* 1991;88:9267–71.
 59. Persico MG, Vincenti V, DiPalma T. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). *Curr Top Microbiol Immunol* 1999;237:31–40.
 60. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994;269:25646–54.
 61. Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R et al. Neuropilin-1 is a placenta growth factor-2 receptor. *J Biol Chem* 1998;273:22272–8.
 62. Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165. *J Biol Chem* 2000;275:18040–5.
 63. DiSalvo J, Bayne ML, Conn G, Kwok PW, Trivedi PG, Soderman DD, et al. Purification and characterization of a naturally occurring vascular endothelial growth factor: placenta growth factor heterodimer. *J Biol Chem* 1995;270:7717–23.
 64. Eriksson A, Cao R, Pawliuk R, Berg SM, Tsang M, Zhou D, et al. Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. *Cancer Cell* 2002;1:99–108.
 65. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernelle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 2001;7:575–83.
 66. Odorisio T, Schietroma C, Zaccaria ML, Cianfarani F, Tiveron C, Tatangelo L, et al. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J Cell Sci* 2002;115:2559–67.
 67. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002;8:831–40.
 68. Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, et al. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res* 2003;92:378–85.
 69. Scholz D, Elsaesser H, Sauer A, Friedrich C, Luttun A, Carmeliet P, et al. Bone marrow transplantation abolishes inhibition of arteriogenesis in placenta growth factor (PlGF) $-/-$ mice. *J Mol Cell Cardiol* 2003;35:177–84.
 70. Clauss M, Weich H, Breier G, Knies U, Röckl W, Waltenberger J, et al. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 1996;271:17629–34.
 71. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285:1182–6.
 72. Ferrara N. Timeline: VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2002;2:795–803.
 73. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–57.
 74. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002;20:4368–80.
 75. Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr Top Microbiol Immunol* 1999;237:97–132.
 76. Chang YS, di Tomaso E, McDonald DM, Jones R, Jain RK, Munn LL. Mosaic blood vessels in

- tumors: frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 2000; 97:14608–13.
77. Hendrix MJC, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer* 2003; 3:411–21.
 78. Borgström P, Bourdon MA, Hillan KJ, Sriramaraio P, Ferrara N. Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors *in vivo*. *Prostate* 1998;35:1–10.
 79. Borgstrom P, Gold DP, Hillan KJ, Ferrara N. Importance of VEGF for breast cancer angiogenesis *in vivo*: implications from intravital microscopy of combination treatments with an anti-VEGF neutralizing monoclonal antibody and doxorubicin. *Anticancer Res* 1999;19:4203–14.
 80. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993;362: 841–4.
 81. Mattern J, Koomagi R, Volm M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. *Br J Cancer* 1996;73:931–4.
 82. Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res* 1996;56: 2013–6.
 83. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993;53:4727–35.
 84. Hartenbach EM, Olson TA, Goswitz JJ, Mohanraj D, Twigg LB, Carson LF, et al. Vascular endothelial growth factor (VEGF) expression and survival in human epithelial ovarian carcinomas. *Cancer Lett* 1997;121:169–75.
 85. Tokunaga T, Oshika Y, Abe Y, Ozeki Y, Sadahiro S, Kijima H, et al. Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 1998;77: 998–1002.
 86. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, et al. Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. *J Clin Invest* 1993;91:153–9.
 87. Toi M, Hoshina T, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994;85:1045–9.
 88. Nomura M, Yamagishi S, Harada S, Yamashita T, Yamashita J, Yamamoto H. Placenta growth factor (PlGF) mRNA expression in brain tumors. *J Neurooncol* 1998;40:123–30.
 89. Hatva E, Bohling T, Jaaskelainen J, Persico MG, Haltia M, Alitalo K. Vascular growth factors and receptors in capillary hemangioblastomas and hemangiopericytomas. *Am J Pathol* 2004;148:763–75.
 90. Viglietto G, Maglione D, Rambaldi M, Cerutti J, Romano A, Trapasso F, et al. Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines. *Oncogene* 1995; 11:1569–79.
 91. Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost* 2003;1:1356–70.
 92. André T, Kotelevets L, Vaillant JC, Coudray AM, Weber L, Prévot S, et al. VEGF, VEGF-B, VEGF-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *Int J Cancer* 2000;86: 174–81.
 93. Hanrahan V, Currie MJ, Gunningham SP, Morrin HR, Scott PA, Robinson BA, et al. The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. *J Pathol* 2003;200:183–94.
 94. Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002;2:38–47.
 95. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxic stimulation of vascular endothelial growth factor expression *in vitro* and *in vivo*. *Lab Invest* 1994;71:374–9.
 96. Shima DT, Adamis AP, Ferrara N, Yeo KT, Allende R, Folkman J, et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Mol Med* 2004;1:182–93.
 97. Ikeda E, Achen MG, Breier G, Risau W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem* 1995;270:19761–6.
 98. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992;359:843–5.

99. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003;3:721-32.
100. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, et al. Molecular mechanisms of transcription activation by HLF and HIF1 in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* 1999;18:1905-14.
101. Bruick RK. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes Dev* 2003;17:2614-23.
102. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;399:271-5.
103. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001;294:1337-40.
104. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem* 2003;278:30772-80.
105. Freedman SJ, Sun ZY, Poy F, Kung AL, Livingston DM, Wagner G, et al. Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. *Proc Natl Acad Sci USA* 2002;99:5367-72.
106. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 2001;15:2675-86.
107. Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, et al. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* 2004;277:26351-5.
108. Eferl R, Wagner EF. AP-1: a double edged sword in tumorigenesis. *Nat Rev Cancer* 2003;3:859-68.
109. Finkenzeller G, Marme D, Weich HA, Hug H. Platelet-derived growth factor-induced transcription of the vascular endothelial growth factor gene is mediated by protein kinase C. *Cancer Res* 1992;52:4821-3.
110. Garrido C, Saule S, Gospodarowicz D. Transcriptional regulation of vascular endothelial growth factor gene expression in ovarian bovine granulosa cells. *Growth Factors* 1993;8:109-17.
111. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem* 1995;270:12607-13.
112. Enholm B, Paavonen K, Ristimäki A, Kumar V, Gunji Y, Klefstrom J, et al. Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 1997;14:2475-83.
113. Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, et al. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 1995;55:4575-80.
114. Grugel S, Finkenzeller G, Weindel K, Barleon B, Marmé D. Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. *J Biol Chem* 1995;270:25915-9.
115. Casanova ML, Larcher F, Casanova B, Murillas R, Fernández-Aceñero MJ, Villanueva C, et al. A critical role for *ras*-mediated, epidermal growth factor receptor-dependent angiogenesis in mouse skin carcinogenesis. *Cancer Res* 2002;62:3402-7.
116. Okada F, Rak JW, St.Croix B, Lieubeau B, Kaya M, Roncari L, et al. Impact of oncogenes in tumor angiogenesis: Mutant K-*ras* up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc Natl Acad Sci USA* 1998;95:3609-14.
117. Kieser A, Weich HA, Brandner G, Marme D, Kolch W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 1994;9:963-9.
118. Mukhopadhyay D, Tsiokas L, Sukhatme VP. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res* 1995;55:6161-5.
119. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1 α . *Genes Dev* 2000;14:34-44.
120. Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS. Effect of p53 status on tumor response to antiangiogenic therapy. *Science* 2002;295:1526-28.
121. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 1993;4:1317-26.
122. Imaizumi T, Itaya H, Nasu S, Yoshida H, Matsubara Y, Fujimoto K, et al. Expression of vascular endothelial growth factor in human umbilical vein endothelial cells stimulated with interleukin-1alpha—an autocrine regulation of angiogenesis and inflammatory reactions. *Thromb Haemost* 2000;83:949-55.

123. Li J, Perrella MA, Tsai JC, Yet SF, Hsieh CM, Yoshizumi M, et al. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. *J Biol Chem* 1995;270:308–12.
124. Pai R, Szabo IL, Soreghan BA, Atay S, Kawanaka H, Tarnawski AS. PGE (2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochem Biophys Res Commun* 2001;286:923–28.
125. Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett* 1995;372:83–7.
126. Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J, et al. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* 2000;89:2637–45.
127. Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM et al. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* 2000;60:1306–11.
128. Iñiguez MA, Rodriguez A, Volpert OV, Fresno M, Redondo JM. Cyclooxygenase-2: a therapeutic target in angiogenesis. *Trends Mol Med* 2003;9:73–8.
129. Green CJ, Lichtlen P, Huynh NT, Yanovsky M, Laderoute KR, Schaffner W, et al. Placenta growth factor gene expression is induced by hypoxia in fibroblasts: a central role for metal transcription factor-1. *Cancer Res* 2001;61:2696–703.
130. Zhang H, Palmer R, Gao X, Kreidberg J, Gerald W, Hsiao L, et al. Transcriptional activation of placental growth factor by the forkhead/winged helix transcription factor FoxD1. *Curr Biol* 2003;13:1625–9.
131. Failla CM, Odorisio T, Cianfarani F, Schietroma C, Puddu P, Zambruno G. Placenta growth factor is induced in human keratinocytes during wound healing. *J Invest Dermatol* 2000;115:388–95.
132. Hauser S, Weich H. A heparin-binding form of placenta growth factor (PIGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 1993;9:259–68.
133. Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, et al. Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *J Biol Chem* 1999;274:35172–8.
134. Gollmer JC, Ladoux A., Gioanni J, Paquis P, Dubreuil A, Chatel M, et al. Expression of vascular endothelial growth factor-b in human astrocytoma. *Neuro-oncol* 2000;2:80–6.
135. Baldwin ME, Stacker SA, Achen MG. Molecular control of lymphangiogenesis. *Bioessays* 2002;24:1030–40.
136. Casley-Smith JR. The fine structure and functioning of tissue channels and lymphatics. *Lymphology* 1980;12:177–83.
137. Roitt I, Brostoff J, Male D. *Immunology*. 5th Edition. London: Mosby International, 1998.
138. Sabin F. On the origin and development of the lymphatic system from the veins and the development of lymph hearts and the thoracic duct in the pig. *Am J Anat* 1902;1:367–89.
139. Wigle JT, Oliver G. *Prox1* function is required for the development of the murine lymphatic system. *Cell* 1999;98:769–78.
140. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for *Prox1* in the induction of the lymphatic endothelial cell phenotype. *EMBO J* 2002;21:1505–13.
141. Oliver G. Lymphatic vasculature development. *Nat Rev Immunol* 2004;4:35–45.
142. Enholm B, Karpanen T, Jeltsch M, Kubo H, Stenback F, Prevo R, et al. Adenoviral expression of vascular endothelial growth factor-c induces lymphangiogenesis in the skin. *Circ Res* 2001;88:623–9.
143. Veikkola T, Jussila L, Mäkinen T, Karpanen T, Jeltsch M, Petrova TV, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001;20:1223–31.
144. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 1997;276:1423–5.
145. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 2004;5:74–80.
146. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt-4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996;15:290–8.
147. Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk-1) and VEGF receptor 3 (Flt-4). *Proc Natl Acad Sci USA* 1998;95:548–53.
148. Baldwin ME, Catimel B, Nice EC, Roufail S, Hall NE, Stenvers KL, et al. The specificity of receptor binding by vascular endothelial growth factor-D is different in mouse and man. *J Biol Chem* 2001;276:19166–71.
149. Kaipainen A, Korhonen J, Mustonen T, van

- Hinsbergh VW, Fang GH, Dumont D, et al. Expression of the *fms*-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995;92:3566-70.
150. Mäkinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 2001;20:4762-73.
 151. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 1998;282:946-9.
 152. Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, et al. A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci USA* 2001;98:12677-82.
 153. Yuan L, Moyon D, Pardanaud L, Bréant C, Karkkainen MJ, Alitalo K, et al. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 2002;129:4797-806.
 154. Witzanbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, et al. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol* 1998;153:381-94.
 155. Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A, Ziche M, et al. *c-fos*-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 1999;96:9671-6.
 156. Byzova TV, Goldman CK, Jankau J, Chen J, Cabrera G, Achen MG, et al. Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns *in vivo*. *Blood* 2002;99:4434-42.
 157. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, et al. Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc Natl Acad Sci USA* 1998;95:14389-94.
 158. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001;159:893-903.
 159. Stacker SA, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J* 2002;16:922-34.
 160. Yanai Y, Furuhashi T, Kimura Y, Yamaguchi K, Yasoshima T, Mitaka T, et al. Vascular endothelial growth factor C promotes human gastric carcinoma lymph node metastasis in mice. *J Exp Clin Cancer Res* 2001;20:419-28.
 161. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, et al. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001;20:672-82.
 162. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001;7:192-8.
 163. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M, et al. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001;61:1786-90.
 164. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 2001;7:186-91.
 165. Kinoshita J, Kitamura K, Kabashima A, Saeki H, Tanaka S, Sugimachi K. Clinical significance of vascular endothelial growth factor-C (VEGF-C) in breast cancer. *Breast Cancer Res Treat* 2001;66:159-64.
 166. Nakamura Y, Yasuoka H, Tsujimoto M, Yang Q, Tsukiyama A, Imabun S, et al. Clinicopathological significance of vascular endothelial growth factor-C in breast carcinoma with long-term follow-up. *Mod Pathol* 2003;16:309-14.
 167. Yonemura Y, Fushida S, Bando E, Kinoshita K, Miwa K, Endo Y, et al. Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer* 2001;37:918-23.
 168. Yokoyama Y, Charnock-Jones DS, Licence D, Yanaihara A, Hastings JM, Holland CM, et al. Vascular endothelial growth factor-D is an independent prognostic factor in epithelial ovarian carcinoma. *Br J Cancer* 2003;88:237-44.
 169. Nakamura Y, Yasuoka H, Tsujimoto M, Yang Q, Imabun S, Nakahara M, et al. Prognostic significance of vascular endothelial growth factor D in breast carcinoma with long-term follow-up. *Clin Cancer Res* 2003;9:716-21.
 170. White JD, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J, et al. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res* 2002;62:1669-75.
 171. Witte MH, Bernas MJ, Martin CP, Witte CL. Lymphangiogenesis and lymphangiodysplasia: From molecular to clinical lymphology. *Microsc Res Tech* 2001;55:122-45.
 172. Ferrell RE, Levinson KL, Esman JH, Kimak MA, Lawrence EC, Barmada MM et al. Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum Mol Genet* 1998;7:2073-8.
 173. Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M. Congenital hereditary lymphedema caused by a mutation that inactivates

- VEGFR3 tyrosine kinase. *Am J Hum Genet* 2000;67:295–301.
174. Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, et al. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. *Am J Hum Genet* 2000;67:1382–8.
 175. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997;16:3898–911.
 176. Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, et al. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem* 1999;274:32127–36.
 177. Siegfried G, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chrétien M, et al. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest* 2003;111:1723–32.
 178. McColl BK, Baldwin ME, Roufail S, Freeman C, Moritz RL, Simpson RJ, et al. Plasmin Activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J Exp Med* 2003;198:863–8.
 179. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002;3:753–66.
 180. Khatib AM, Siegfried G, Chretien M, Metrakos P, Seidah NG. Proprotein convertases in tumor progression and malignancy: novel targets in cancer therapy. *Am J Pathol* 2002;160:1921–35.
 181. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev* 1995;9:794–807.
 182. Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, et al. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J* 2000;14:2087–96.
 183. Achen MG, Williams RA, Minekus MP, Thornton GE, Stenvers K, Rogers PAW, et al. Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis. *J Pathol* 2001;193:147–54.
 184. Bugge TH, Kombrinck KW, Xiao Q, Holmback K, Daugherty CC, Witte DP, et al. Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. *Blood* 1997;90:4522–31.
 185. Valtola R, Salven P, Heikkilä P, Taipale J, Joensuu H, Rehn M, et al. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999;154:1381–90.
 186. Ristimäki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem* 1998;273:8413–18.
 187. Su JL, Shih JY, Yen ML, Jeng YM, Chang CC, Hsieh CY, et al. Cyclooxygenase-2 induces EP1- and HER-2/Neu-dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. *Cancer Res* 2004;64:554–64.
 188. Pichiule P, Chavez JC, LaManna J.C. Hypoxic regulation of angiopoietin-2 expression in endothelial cells. *J Biol Chem* 2003; Epub ahead of print.
 189. Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, et al. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 2002;3:411–23.
 190. Hida T, Yatabe Y, Achiwa H, Murmatsu H, Kozaki K, Nakamura S, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 1998;58:3761–4.
 191. Orlandini M, Marconcini L, Ferruzzi R, Oliviero S. Identification of a *c-fos*-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. *Proc Natl Acad Sci USA* 1996;93:11675–80.
 192. Jenkins NA, Woollatt E, Crawford J, Gilbert DJ, Baldwin ME, Sutherland GR, et al. Mapping of the gene for vascular endothelial growth factor-D in mouse and man to the X chromosome. *Chromosome Res* 1997;5:502–5.
 193. Rocchigiani M, Lestingi M, Luddi A, Orlandini M, Franco B, Rossi E, et al. Human *FIGF*: cloning, gene structure, and mapping to chromosome Xp22.1 between the *PIGA* and the *GRPR* genes. *Genomics* 1998;47:207–16.
 194. Debinski W, Slagle-Webb B, Achen MG, Stacker SA, Tulchinsky E, Gillespie GY, et al. VEGF-D is an X-linked/AP-1 regulated putative onco-angiogen in human glioblastoma multiforme. *Mol Med* 2001;7:598–608.
 195. Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. *Ann Rev Cell Dev Biol* 2003;19:207–35.
 196. Orlandini M, Oliviero S. In fibroblasts *Vegf-D* expression is induced by cell-cell contact mediated by cadherin-11. *J Biol Chem* 2001;276:6576–81.
 197. Orlandini M, Semboloni S, Oliviero S. Beta-catenin inversely regulates vascular endothelial growth factor-D mRNA stability. *J Biol Chem* 2003; 278:44650–6.

Invited Review

Angiogenesis: an update

L. Díaz-Flores, R. Gutiérrez and H. Varela

Department of Anatomy, Histology and Pathology, Faculty of Medicine, University of La Laguna, Canary Islands, Spain

Summary. Angiogenesis is the neovascularization or formation of new blood vessels from the established microcirculation. It is particularly important and indispensable in a large number of normal and pathological processes during pre- and post-natal life, including neoplasia, inflammation, wound repair and collateralization in response to ischemic stimuli. The current interest in the role of neovascularization in the transition from hyperplasia to neoplasia, as well as in the tumour growth and metastasis, has brought about a large number of studies on angiogenesis. The complex processes of neovascularization, quiescent in the adult organism, may occur rapidly in several circumstances, with the implication of the following events: a) endothelial cell (EC) and pericyte activation; b) basal lamina degradation; c) migration and proliferation of EC and pericytes; d) formation of a new capillary vessel lumen; e) appearance of pericytes around the new capillaries; f) development of a new basal lamina; g) capillary loop formation; h) persistence or involution, and differentiation of the new vessels; and i) capillary network formation and, eventually, organization into larger microvessels. The use of numerous "in vivo" and "in vitro" systems has facilitated the assessment of angiogenesis control, in which angiogenic (fibroblast growth factors, vascular endothelial growth factor, platelet endothelial growth factor, E series prostaglandin, angiogenin, monobutyrin) and antiangiogenic (cartilage-derived angiogenic inhibitor, thrombospondin, protamine, platelet factor-4, interferon, angiostatic antibiotics, steroids) substances intervene. Heparin and heparin sulphate also play a key role in these mechanisms. A greater knowledge of angiogenesis control may lead to the development of a potential therapy in angiogenesis-related processes.

Key words: Angiogenesis, Endothelium, Pericytes, Tumour angiogenesis, Angiogenic factors, Angiogenic antagonists

Offprint requests to: Prof. Lucio Díaz-Flores, Departamento de Anatomía, Anatomía Patológica e Histología, Facultad de Medicina, Universidad de la Laguna, Tenerife, Islas Canarias, Spain

Processes in the development of new vessels. Vasculogenesis and angiogenesis

In the development of new blood vessels, it is necessary to distinguish between vasculogenesis and angiogenesis (Risau et al., 1988; Risau and Lemmon, 1988; Demir et al., 1989). Vasculogenesis is the process by which some vessels develop in the embryo, such as the dorsal aortae and the posterior cardinal veins. Histogenically, vasculogenesis is defined as capillary development from differentiating endothelial cells (EC) "in situ". This process takes place in blood islands, which originate from the splanchnopleuric mesoderm. After their segregation from the mesoderm, some mesenchymal cells transform into nests of isolated cell cords of hemangioblasts, which are the precursors of both EC and blood cells. The peripheric cells of the angioblastic masses differentiate into EC, while the blood cell precursor cells are found in the centre of the blood islands, wherein a lumen is developed. The newly formed lumens soon coalesce (Gonzalez-Crussi, 1971; Pardanaud et al., 1987; Coffin and Poole, 1988). Finally, the smooth muscle cells and pericytes are aggregated from undifferentiated mesenchyme.

Angiogenesis is the process characterized by the formation of new blood vessels from an established microvasculature during the development of the embryonic vascular tree as well as in several normal and pathological conditions during post-natal life. Histogenically, it is defined as a mechanism of neovascularization by the sprouting of capillaries from pre-existing vessels.

In the present article, only the angiogenesis process will be reviewed.

Incidence and importance of angiogenesis

Angiogenesis is indispensable in embryonic and fetal development, as well as in a large number of normal and pathological processes during post-natal life (Warren, 1979a,b; Schor and Schor, 1983; Folkman, 1985; D'Amore and Thompson, 1987; Madri and Pratt, 1988; Paweletz and Knierim, 1989). Therefore, the area

involved in the study of angiogenesis is far-reaching, covering numerous fields and many disciplines (Auerbach et al., 1991).

During early embryonic development, new vessel formation or neovascularization, is an important event (Feinberg et al., 1991). Thus, a wide range of investigations at cellular and molecular levels on embryonic angiogenesis during organogenesis exist (Stewart and Wiley, 1981; Eklom et al., 1982; Risau, 1986; Risau and Eklom, 1986; Risau and Lemmon, 1988; Risau et al., 1988).

Angiogenesis is generally a quiescent process in the adult organism. Nevertheless it may occur rapidly in several normal circumstances. For example, the need for additional vasculature is imposed on the cyclic evolution of transient structures in the female reproductive system (for review, see Findlay, 1986), including the sequential maturation of ovarian pre-ovulatory follicles (Harris and Eakin, 1949; Richards, 1980; Sato et al., 1982; Koos and LeMaire, 1983; Makris et al., 1984; Frederick et al., 1984; Rone and Goodman, 1985; Koos, 1986) and subsequent development of the corpora lutea (Brambell, 1956; Jakob et al., 1977; Heder et al., 1979; Kanzani et al., 1985; Goodman and Rone, 1985), cyclic extensions and repair of the functional endometrium (Markee, 1940; Abell, 1946; Foley et al., 1978; Christianens et al., 1982), decidual transformation, implantation and placentation (Edwards, 1980; Gospodarowicz et al., 1985; Feinberg et al., 1991), and mammary gland changes associated with lactation.

Furthermore, angiogenesis is an important component of many pathological processes, such as chronic inflammation, regeneration, wound healing, thrombolytic organization, collateral circulation development, neoplasias, and of several conditions in which the term "angiogenic disease" has been proposed, since an abnormality of capillary growth is their principal pathological feature (Folkman, 1989). Among the "angiogenic diseases" are hemangiomas, psoriasis (Majewski et al., 1987), scleroderma (Kaminski et al., 1984), rheumatoid arthritis, diabetic retinopathy (Davis, 1988) and neovascular glaucoma.

The role of angiogenesis in neoplasias is of great interest, especially in the progressive growth and metastases of solid tumours (Folkman, 1972; Schor and Schor, 1983; Folkman, 1985a,b,c). This has brought about a large number of descriptive studies on angiogenesis, as well as studies on the possible mechanisms involved in this process (Folkman, 1975, 1985a,b,c; D'Amore and Thompson, 1987; Folkman and Klagsbrun, 1987; Zetter, 1988; Pawletz and Knierim, 1989; Blood and Zetter, 1990).

Angiogenic phenomena

The regions of the vascular tree with angiogenic capacity, the timing, pattern and the events of new blood vessel formation, and some of the peculiar forms of angiogenesis, will be considered in this section.

Regions of vascular tree with angiogenic capacity

In general, it is accepted that vascular sprouts originate from the walls of pre-existing capillaries and from small venules (Mc Cracken et al., 1979; Tano et al., 1981; Sholley et al., 1984), but mainly from the latter (Schoefl, 1963; Ausprunk and Folkman, 1977). Some authors restrict the origin and point out that new vessels arise only from the venous side of the circulation (Phillips et al., 1991), specifically from small venules which lack smooth muscle cells (Ausprunk and

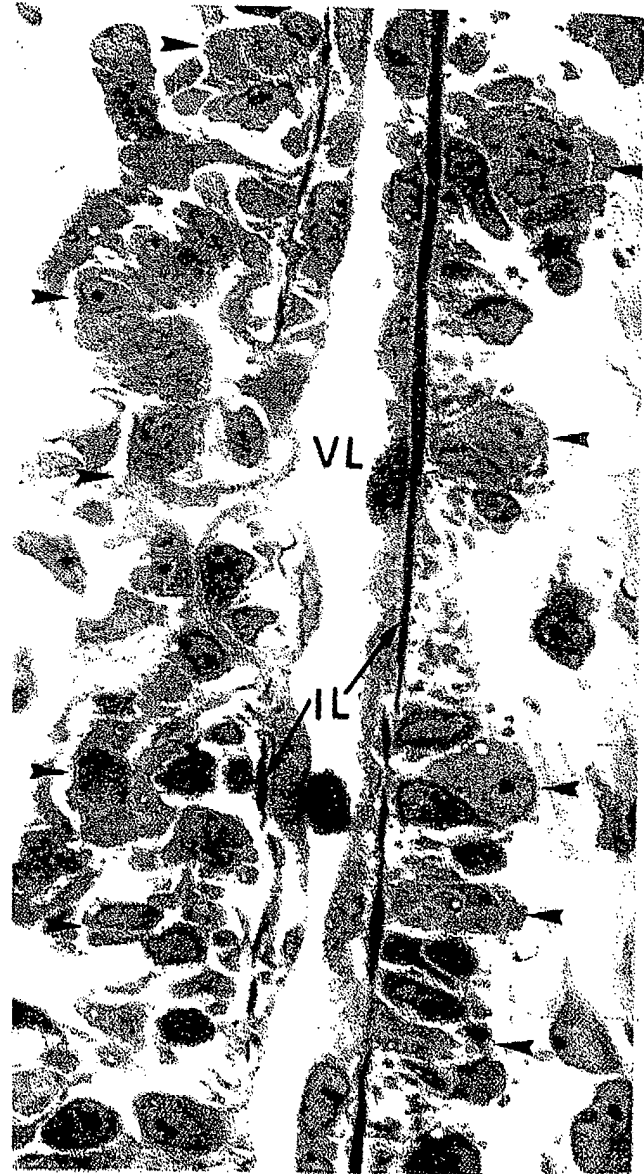


Fig. 1. Femoral vein of the rat 5 days after PGE₂ and triacetin administration into the soft tissue surrounding it. Numerous vascular buds arising from the vein are present (arrowheads). VL: Vein lumen; IL: discontinuous internal elastic lamina. Semithin section; toluidine blue. x 900

...s and
...et al.
...latter
...Some
...vessels
...lation
...enules
...k and

Folkman, 1977). For others, the fact that the venules were the predominant source, perhaps only reflects the larger surface area of the venular plexus in certain explored areas (Burger et al., 1983). Recently, it has been demonstrated that vessels of greater calibre in the venous side of the circulation, such as the rat femoral vein, with a discontinuous internal elastic lamina and smooth muscle cells in their media layer, are capable of contributing to angiogenesis, on occasions with an intense neovascularization (Fig. 1). Indeed, a single application of prostaglandins E1 and E2 in triacetin solution into the soft tissue surrounding the rat femoral vein induces a sudden and intense angiogenesis, with the vascular sprouts arising from the EC in the intima of the vein (Diaz-Flores et al., 1994).

Although few ^3H -labelled EC in small arteries have been demonstrated during angiogenesis (Burger and Klintworth, 1981), new vessels do not seem to originate from the arterial side of the circulation.

Timing and pattern of capillary formation. Vision of angiogenesis using scanning electron microscopy

Scanning electron microscopy of vascular casts has been utilized to study the early changes in vasculature responding to angiogenic stimuli (Burger et al., 1983; Garbett and Gibbins, 1987; Forsman and McCormack, 1992). During the first hours, preformed capillaries and postcapillary venules became widely distended and tortuous, the postcapillary vein being affected more extensively. In the walls of the venules, numerous impressions occur, corresponding to marginating leukocytes. Vascular sprouts originate from both venules and capillaries, although predominantly from venules. The sprouts appear rapidly, being seen as early as 27 hours after angiogenic stimuli (Burger et al., 1983). Between three and five days, the number of sprouts is intensely increased to produce a rich anastomosing plexus. In some conditions, the process of neovascularization may be extremely fast. For example, throughout days 1 and 2 of the cycle, the hamster corpus luteum vasculature grows from the existing theca vessels. Flat veins, characteristic of the external surface of a mature corpus luteum, appear at day 2. Involution of the corpus luteum vasculature takes place throughout day 3 of the cycle (Forsman and McCormack, 1992).

Events of new blood vessel formation

Angiogenesis is a multistep complex process which has been considered in several studies under various pathological conditions (Cliff, 1963; Schoefl, 1963; Yamagami, 1970; Bar and Wolff, 1972; Cavallo et al., 1973; Ausprunk and Folkman, 1977; Warren, 1979; Sholley et al., 1984; Dvorak et al., 1987; Wakui et al., 1988; Paku and Paweletz, 1991). Besides the inflammatory phenomena which occur prior to and during angiogenesis, the events essentially involved in capillary growth in vivo include (Brenk, 1955; Cliff,

1963; Crocker et al., 1970; Yamagami, 1970; Inomata et al., 1971; Ausprunk and Folkman, 1977; Schor and Schor, 1983; Sholley et al., 1984; Folkman, 1985a,b,c; Freemont and Ford, 1985; Furcht, 1986; D'Amore and Thompson, 1987; Madri and Pratt, 1988; Paweletz and Knierim, 1989; Paku and Paweletz, 1991; Diaz-Flores et al., 1992): a) EC and pericyte activation; b) degradation of the basal lamina of pre-existing vessels by EC (proteolytic destruction of the extracellular matrix); c) EC migration from pre-existing vessels towards the angiogenic stimulus; d) EC proliferation; e) migration and proliferation of pericytes from pre-existing vessels; f) formation of a new capillary vessel lumen (vascular



Fig. 2. Preformed postcapillary venule of rat surrounding epineural microcirculation after angiogenesis stimulus and intravenous administration of Monastral Blue (MB). MB particles have leaked through interendothelial gaps and are present between the endothelial cells (EC) and the pericytes (P), within the basal lamina. Ultrathin section; uranyl acetate and lead citrate. x 8,000

tube formation); g) appearance of pericytes around the new capillaries (pericytes in angiogenesis); h) changes in extracellular matrix with development of a new basal lamina; i) capillary loop formation; j) early changes in the newly formed vessels (persistence, involution and differentiation); and k) capillary network formation and eventually organization of larger microvessels.

Although, stepwise, the current model of angiogenesis is controversial, needing reconsideration, (Schlingemann et al., 1991), our review will follow the latter, referring to the doubts therein and including the associated inflammatory phenomena.

Inflammatory phenomena associated with angiogenesis:

New blood vessel formation is an orderly process which is coordinated with inflammation, immunological activity, debridement and fibroplasia (Jennings and Florey, 1970; Auerbach, 1981; Peacock, 1984; West et al., 1985). Thus, an inflammatory response, with vascular dilation, increased vascular permeability, and diapedesis of leukocytes may precede and accompany the angiogenic phenomena (Mc Cracken et al., 1979).

Dilation of the blood venules and capillaries occurs rapidly. For example, in corneal vascularization induced

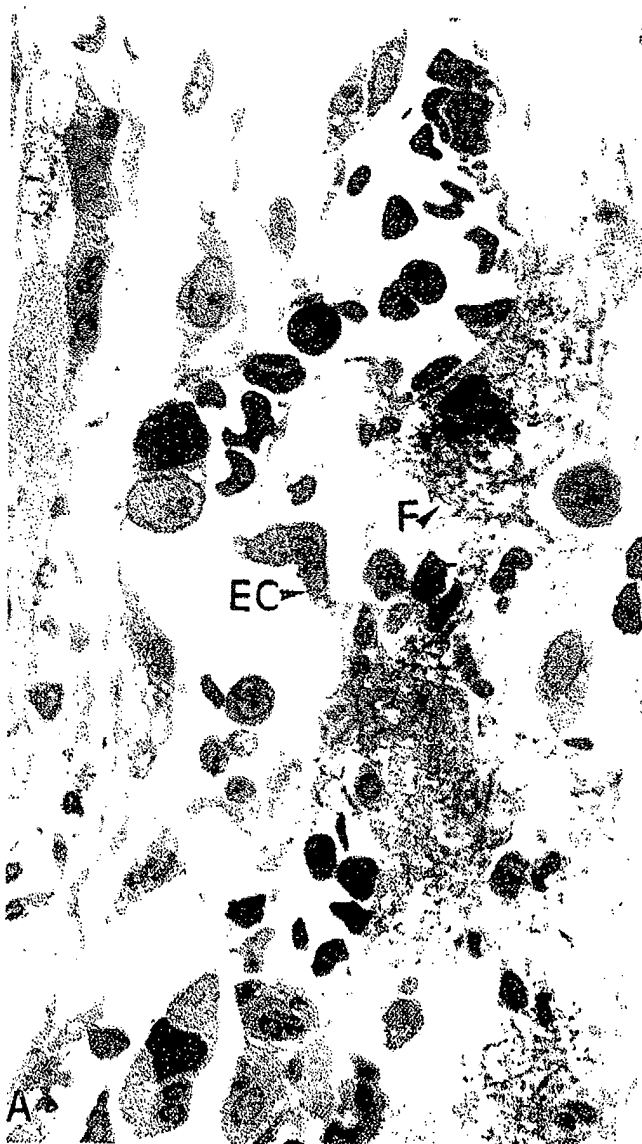


Fig. 3. The increased vascular permeability during angiogenesis is demonstrated by the presence of extravascular fibrin (F) around both a parent venule (A) and an advanced border of a new vessel (B) (arrowheads). EC: Endothelial cell; P: Pericyte. A) Semithin section. x 1,100. B) Ultrathin section; uranyl acetate and lead citrate. x 12,000

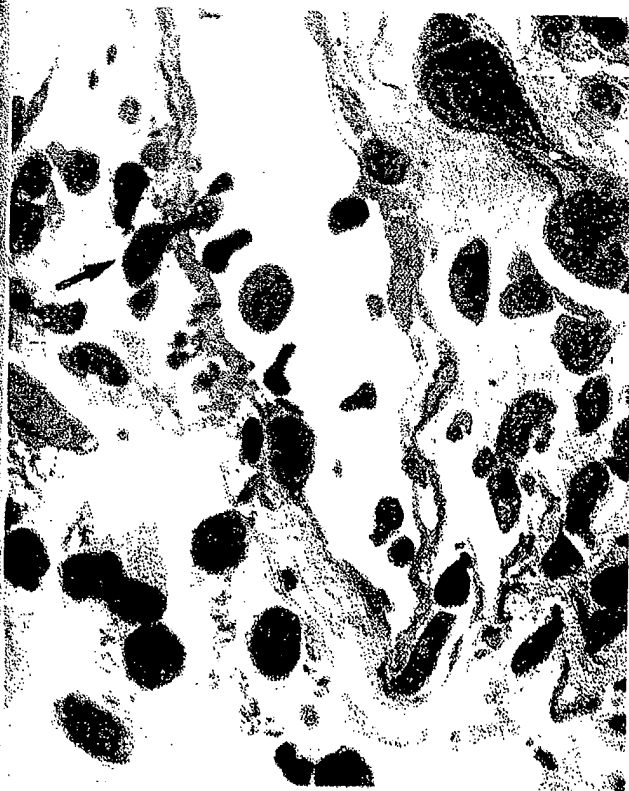
Fig. 4. vasculature (predominant through section;

ngio- by silver nitrate, the dilation takes place within 1 hour (Mc Cracken et al., 1979), intensifying progressively. The connective tissue becomes edematous and intercellular contacts between EC have been described as opened (Ausprunk and Folkman, 1977; Dvorak et al., 1988; Paku and Paweletz, 1991).

process
ogical
and
est et
with
, and
pany
).
ccurs
luced

The changes in vascular permeability during angiogenesis were studied using the intravenous injection of different markers (Schoefl, 1963; Sugiura and Matsuda, 1969; Yamagami, 1970; Garbett and Gibbins, 1987), such as colloidal carbon or monastral blue to label leaky vessels (Garbett and Gibbins, 1987; Diaz-Flores et al., 1992) (Fig. 2). Preformed capillaries and post-capillary venules become variably permeable to the introduced colloid marker, the observed pattern being different from the characteristic labelling pattern produced by the inflammatory mediator, histamine (Garbett and Gibbins, 1987). During angiogenesis, the interconnecting networks of newly-formed vessels often label strongly, especially along the advancing border of new vessels, until the basal lamina is laid down and pericytes emerge along the length of the sprout (Schoefl, 1963). The increased vascular permeability is also demonstrated by the presence of extravascular fibrin (Fig. 3) and dilated lymphatics. Extravascular fibrin

deposits, which are found during inflammation, wound healing (Madri and Pratt, 1988) and in the periphery of tumours (Nagy et al., 1988), are important during angiogenesis. Thus, neovascularization is induced by fibrin gels in vitro (Nicosia et al., 1982) and in vivo (Dvorak et al., 1987). Also, it has been demonstrated that cultured EC synthesize fibronectin (Jaffe and Mosher, 1978; Birdwell et al., 1978) and that the growing capillaries produce fibronectin in situ (Clark, 1982a,b). During EC growth, the fibronectin may



parent
lirathin

Fig. 4. Dilated perilimbal venule in the first stage of corneal vascularization induced by silver nitrate in the rat. Inflammatory cells (predominantly polymorphonuclear leukocytes) are seen passing through the endothelial junction (arrow) and in the interstitium. Semithin section; toluidine blue. x 700

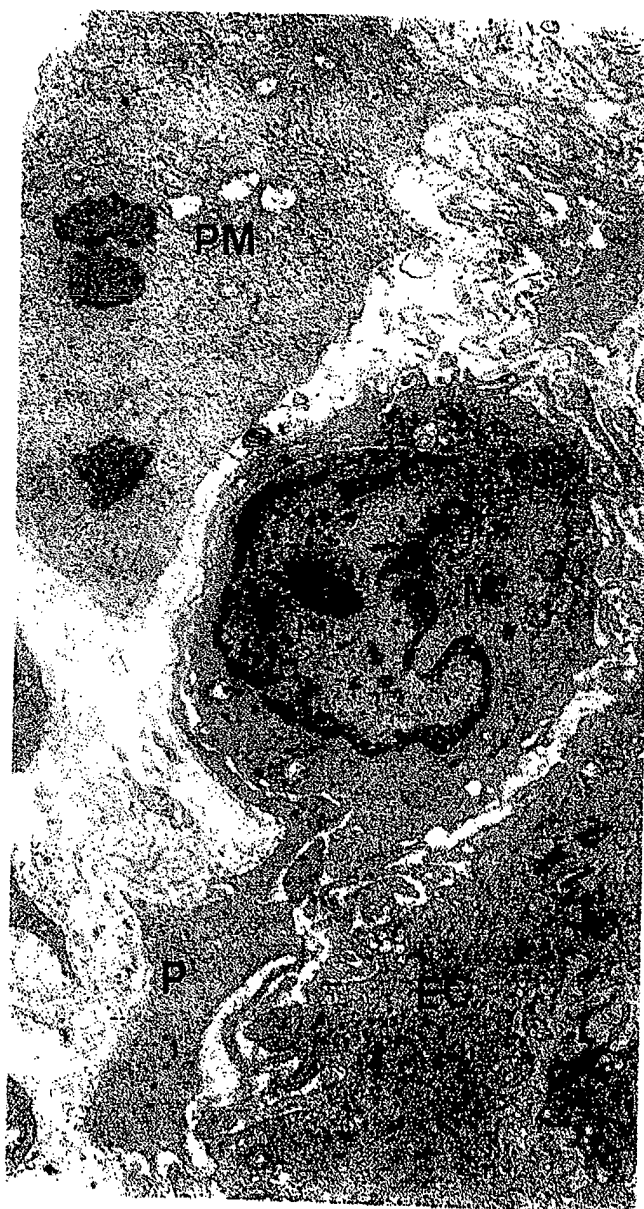


Fig. 5. Preformed postcapillary venule after angiogenic stimulus. A macrophage (M) appears trapped between endothelial cells (EC) and pericytes (P), within the basal lamina. A pericyte in mitosis (PM) is observed. L: Postcapillary venule lumen. Ultrathin section; uranyl acetate and lead citrate. x 13,500

mediate EC adherence (Clark, 1985) and chemotaxis (Bowersox and Sorgente, 1982).

Within a few hours, intravascular accumulation of platelets and polymorphonuclear leukocytes (PMNs) occurs, with early diapedesis of leukocytes outside the vessel lumen. This association of inflammatory cells with the neovascularization in several processes is a recognised observation (Clark and Clark, 1939; McDonald, 1959; Grillo, 1963; Jennings and Florey, 1970; Ross et al., 1970; Ryan and Spector, 1970). Indeed, before and during vascular sprouting, inflammatory cells are observed adhering to the endothelium of the parent vessels, as well as passing through the endothelial junctions and the pericyte-endothelial space. Between 1 and 6 h after angiogenic stimuli, the PMNs predominate (Fig. 4). Thereafter, the number of monocytes/macrophages increases, while the number of PMNs decreases dramatically. Frequently, the monocytes/macrophages, either individually or in small clusters of two or three, simultaneously appear trapped between the EC and the pericytes, within the basal lamina (Fig. 5). The new vessels arise following the margination and diapedesis of the leukocytes. In an immunohistochemical study of the cellular events after chemical



Fig. 6. Bulging pericytes (P) from a limbal postcapillary venule with shortening of their processes (arrows). Semithin section; toluidine blue. $\times 900$

cauterization of the murine cornea, it has been demonstrated that the infiltrating cells which preceded the ingrowth of new blood vessels are granulocytes and inflammatory monocytes. On the contrary, macrophages, T lymphocytes, eosinophils, or mast cells were not part of the infiltrate before the appearance of new capillaries (Sunderkötter et al., 1991). Leukocytes do not seem to be essential for the initiation and continuation of angiogenesis. For example, corneal vascularization has been produced in the absence of leukocytes in rats and rabbits (Sholley et al., 1978). Nevertheless, leukocytes may have a facilitatory or augmentative role in vascularization (Fromer and Klintworth, 1975a,b, 1976; Polverini, 1977a,b; Sholley et al., 1978). Progressively, fibrin material, erythrocytes, macrophages and dividing fibroblasts appear in the interstitium. Usually, angiogenesis is also accompanied, to a variable extent, by fibroblast proliferation which participates in reparative processes.

Endothelial cell and pericyte activation:

The earliest morphological changes in the normally quiescent EC consist of hypertrophy with bulging in the vascular lumen, nuclear enlargement, nucleolar prominence, dispersal of the ribosomes into their free form, increase in the number of organelles and the formation of projections from their surfaces (Schoefl, 1963; Yamagami, 1970; Ausprunk and Folkman 1977; McCracken et al., 1979; Burger and Klintworth, 1981; D'Amore and Thompson, 1987). Increased endothelial DNA-synthesis beginning at the onset of angiogenesis has been described (Burger and Klintworth, 1981; Burger et al., 1983). Proteases such as metalloproteases and plasminogen activators are secreted from sprouting EC (Pepper et al., 1990).

The pericytes also display modifications of both their morphological characteristics and topographic relationship (Diaz-Flores et al., 1992) (Fig. 6). The first ultrastructural changes in the enlarged pericytes from post-capillary venules include the shortening of their processes and an increase in the number of cytoplasmic polyribosomes (Fig. 7) (McCracken et al., 1979) (see pericytes in angiogenesis).

Basal lamina degradation:

The fragmentation and disappearance of the basal lamina is a necessary step for the EC migration from the mother vessels (Cliff, 1963; Schoefl, 1963; Ausprunk and Folkman, 1977). Complete disintegration occurs on the side closest to the angiogenic stimulus, coinciding with those areas wherein the EC start to grow outwards, while subtle alterations seem to appear around the whole circumference of the parent vessel. Therefore, the EC sprouts do not have basal lamina, but a homogeneous provisional substratum with altered proteoglycans (Clark et al., 1982a,b). The changes in the original basal lamina

are due by the t al., 198 release response EC "in v

Endo:

Tradit by mean. EC migr during at the initia

Fig. 7. Prefon bulging pericy cytoplasmic r between the f lumen. Ultrathir

are due to proteolytic enzymes synthesized and secreted by the activated EC (Rifkin et al., 1982; Montesano et al., 1986; Moscatelli and Rifkin, 1988). Indeed, the release of plasminogen activator and collagenase, in response to angiogenic factors, has been demonstrated in EC "in vitro" (Rifkin et al., 1982).

Endothelial cell migration:

Traditionally, it is considered that blood vessels grow by means of a movement of EC (His, 1868). This fact of EC migration is currently considered an important step during angiogenesis (Ausprunk and Folkman, 1977). In the initial phase of neo-vascularization, the EC degrade

the vascular basement membrane of the parent vessel, protrude through its wall and begin to migrate into the interstitial space towards the angiogenic stimulus. Most researchers agree that these changes precede endothelial replication in such a way that migration and mitoses are independent phenomena (Sholley et al., 1977a,b; Wall et al., 1978). In other words, angiogenesis begins with pseudopodia of migrating EC and progresses to the proliferation of these cells (Matsushashi, 1961, 1962; Sugiura and Matsuda, 1969; Yamagami, 1970; McCracken et al., 1979). Therefore, angiogenic stimuli may operate through chemotaxis and EC mitosis may be a secondary event (Sholley et al., 1977a,b; Folkman, 1982). When the entire EC migrates into the interstitium,



Fig. 7. Preformed postcapillary venule after angiogenic stimulus. A bulging pericyte (P) with increase in its size and in the number of cytoplasmic polyribosomes is seen. A macrophage (M) appears between the pericyte and endothelium (E). L: postcapillary venule lumen. Ultrathin section; uranyl acetate and lead citrate. x 13,500



Fig. 8. Endothelial cell migration during angiogenesis. Bicellular or bipolar configuration. Two endothelial cells (arrows) migrate from the wall of the parent vessel towards the perivascular space. L: Parent vessel lumen; ultrathin section. Uranyl acetate and lead citrate. x 13,500

other EC follow and loose EC sprouts or cords are formed in the perivascular stroma. Two different types of EC migration have been described (Paku y Paweletz, 1991): a) bicellular or bipolar configuration (Burger et al., 1983; Folkman, 1984; Wakui, 1988), also termed as telescoping formation (Sholley et al., 1984); and b) linear formation with a single cell type (Folkman, 1986). In the bicellular configuration (Fig. 8), two or more EC migrate from the wall of the parent vessel towards the perivascular space, forming nearly parallel processes (Sholley et al., 1984; Wakui, 1988). The pair of EC, attached to each other, with numerous free polyribosomes and abundant intermediate filaments, may appear in an area embedded within the wall of the parent vessel, while their processes extend outwards in unison to form the endothelial sprout with a narrow slit-like lumen (Wakui, 1988). A giant dense body, has been found in the cells forming the endothelial sprouts (Furusato et al., 1984; Wakui, 1988). In the linear formation (Fig. 9), a single EC projection and/or pseudopod migrates into the surrounding connective tissue from the parent capillaries (Ausprunk and Folkman, 1977; Folkman et al., 1979; Furusato et al.,

1984; 1985; Folkman, 1984, 1986). In both cases of migration, the EC aligned with one another to create a solid sprout, or with intercellular slit-like lumina. The elongation and proliferation of the EC progressively lengthens the sprout.

During the process in which the EC protrude and the vascular basement membrane is degraded, microscopic bleeding may occur.

The presence of abundant contractile intermediate filaments in the endothelium of the sprouts might be important for the extension and migration of these sprouts (Furusato et al., 1985; Wakui, 1988). Some authors have reported the loosening of intercellular contacts between migrating EC with other EC of the mother vessels, suggesting that the disrupted cell junctions may release EC from contact inhibition and allow proliferation, while producing an increased vascular permeability (Ausprunk and Folkman, 1977). On the contrary, other investigators have never observed opened intercellular contacts in the neighbourhood of new capillaries, and are of the opinion that pre-existing intercellular junctions contribute to a parallel movement of the EC and preserve the inside-outside polarity of the

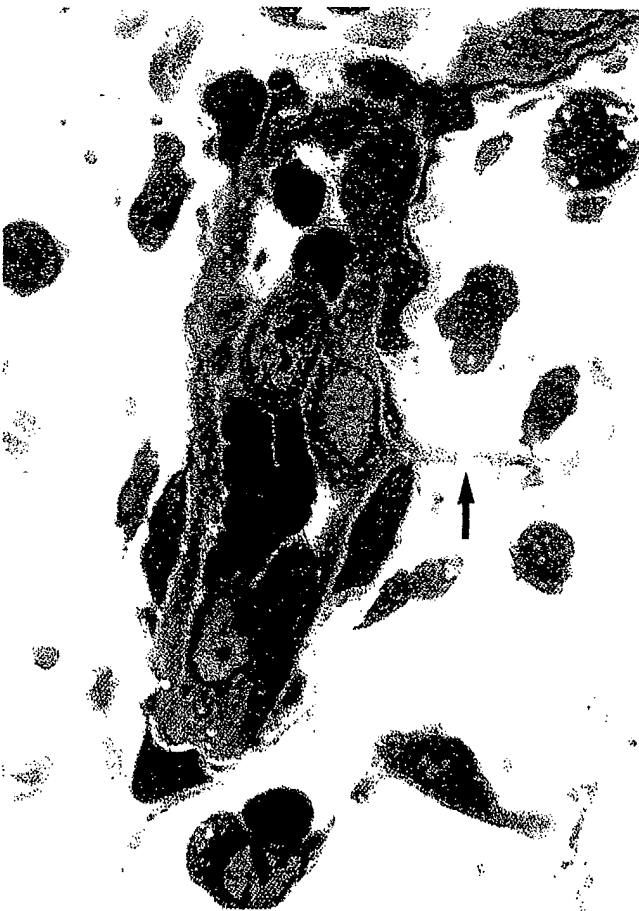


Fig. 9. Endothelial cell migration during angiogenesis. Linear formation. A single EC projection or pseudopod (arrows) migrates into the interstitium from the parent vessel. Semithin sections; toluidine blue, x 900

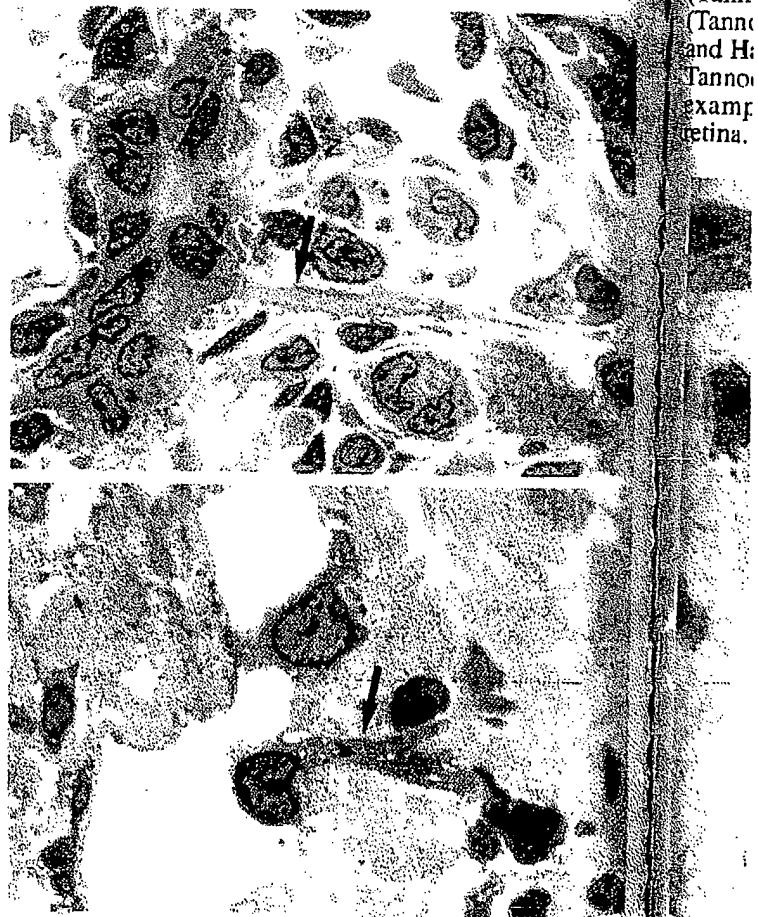


Fig. 10. Advanced linear formation. Multiple EC projections (arrows) migrate into the interstitium from the parent vessel. Semithin sections; toluidine blue, x 900

ses of teate a. The sively nd the scopio

mediate ght be these Some llular of the d cell on and eased 1977) served od of xisting ement of the

nd the scopio
mediate ght be these
Some llular of the d cell
on and eased 1977)
served od of xisting
ement of the

sprouting EC (Paku and Paweletz, 1991).

The chemotactic behaviour of EC at the tips of growing vessels is facilitated by the secretion of plasminogen activator and collagenases (Moscatelli et al., 1981).

The EC migration, in response to the extracellular matrix, depends on the integrin family of cell adhesion receptors (Leavesley, 1993). Indeed, attachment, spreading and migration of EC are mediated by integrins α 2B1 and α 4B3 (Leavesley, 1993). EC migration on collagen and vitronectin is mediated by α 4B3 and it occurs in a calcium-dependent manner, while collagen recognition by α 2B1 promotes EC migration in the absence of calcium (Leavesley, 1993).

Endothelial cell proliferation:

Mature endothelial cells, normally in a resting state, show an extremely slow turnover rate (Algire et al., 1945; Altschul, 1954; Sparagen et al., 1962; Folkman and Cotran, 1976) of 2 months or more. Thus, using ^3H -Thymidine, the labelling index is lower than 1% in normal capillary and venular EC of the retina, liver (Tannock and Hayashi, 1972), myocardium, stomach (Tannock and Hayashi, 1972), striated muscle (Tannock and Hayashi, 1972) and skin (Cavallo et al., 1972, 1973; Tannock and Hayashi, 1972; Polverini et al., 1977b). For example, it is 0.01% in capillary EC in the adult rat retina. Since the turnover rates of EC are extremely low,

angiogenesis is generally a quiescent process in the healthy adult organism (Shweiki et al., 1993). Nevertheless, the EC can quickly convert to a proliferative state during angiogenesis and in several related processes, such as endothelium repopulation in organ transplants, repair of large vessel defects and thrombi recanalization (Cavallo et al., 1973; Folkman, 1984). However, EC proliferation is not absolutely essential, since angiogenesis has been shown to take place even in the absence of EC replication (Sholley et al., 1984).

During angiogenesis, endothelial DNA synthesis occurs in parent vessels before sprouting (Fig. 10), and according to some authors as early as 6 to 8 hours after an angiogenic stimulus is applied (Cavallo et al., 1973). The increase of the turnover rate of EC can be considerable. For example, the ^3H -Thymidine labelling index of EC increases to 9% in tumours (Denekamp and Hobson, 1982). The time and the exact site of EC division are controversial. For some investigators, EC mitosis appear concomitant with sprouting (Sholley et al., 1984), while most authors are of the opinion that EC begin in mitosis after they start to migrate. The EC mitosis appear in both the parent vessels (Fig. 11) and the newly formed vessels. In the latter, it has been pointed out that they occur at the tip (Clark and Clark, 1939; Hadfield, 1951), but it is accepted that when capillary sprout budding begins, endothelial proliferation takes place in cells following the "leader EC", but not

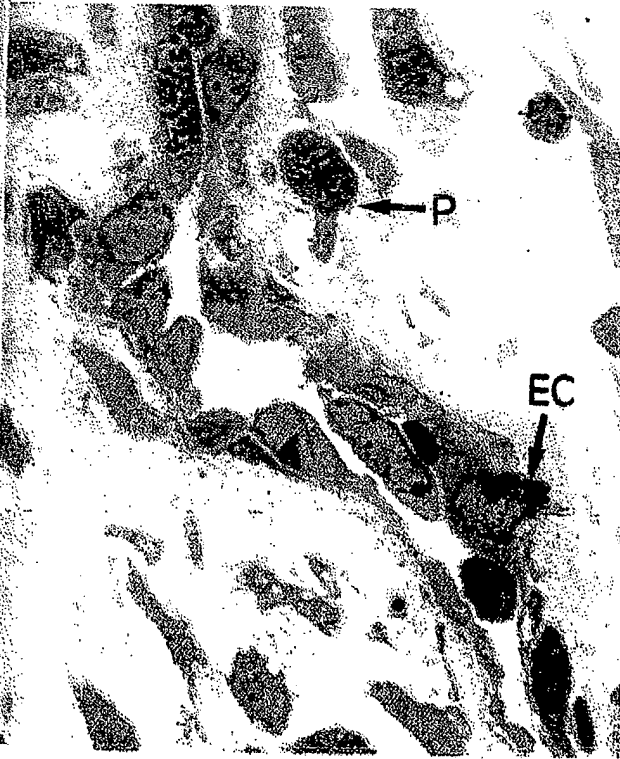


Fig. 10. Autoradiograph of a preformed postcapillary venule after angiogenic stimulus. ^3H -labelled endothelial cells (EC) and pericytes (P) are present. An EC in mitosis (M) is observed. Semithin sections; toluidine blue. $\times 1,150$

usually at their tips. In other words, the zone of replication is closer to the parent vessel (Cliff, 1965; Ausprunk and Folkman, 1977; Folkman, 1982, 1986; Clark, 1985).

The ability of angiogenic stimuli to induce replication in confluent EC is associated with disruption of cell-cell contacts (Bavisotto et al., 1990). Likewise, the replicative state and its ability to respond to endogenous mitogens may depend on cytoskeletal organization, such as microtubule destabilization or changes in the cell shape (Liaw and Schwartz, 1993). Finally, the collagen in the interstitium seems to have an influence on EC proliferation (Madri and Stenn, 1982; Schor et al., 1983).

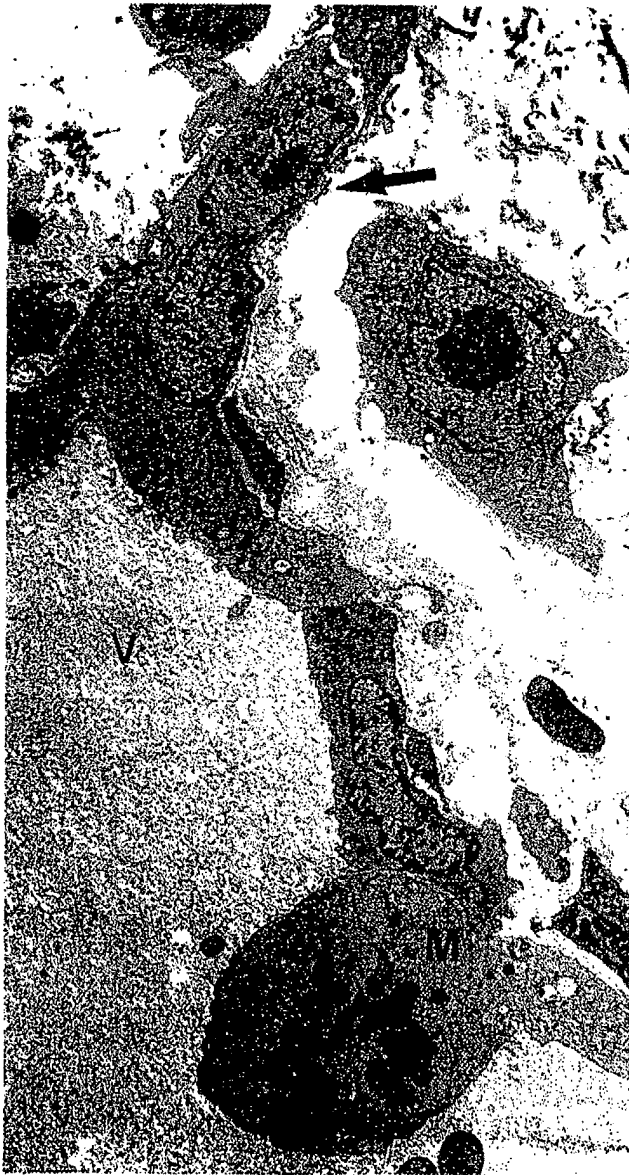


Fig. 11. A venule (V) and its capillary sprout (arrow) are shown. An endothelial cell in mitosis (M) is observed in the parent vessel. Ultrathin section; uranyl acetate and lead citrate. x 13,500

Migration and proliferation of pericytes from pre-existing vessels (see Pericytes in angiogenesis):

Formation of the new capillary vessel lumen. During angiogenesis, the endothelial cells form tubular channels capable of carrying blood. Two distinct types have been considered in the formation of the new capillary vessel lumen (Wagner, 1980): a) previous intracellular vacuolization in the endothelial cytoplasm of contiguous cells which leads to intercellular canalization by connection of the vacuoles (Sabin, 1920; Folkman and Haudenschild, 1980; Furusato et al., 1984, 1985); and b) initial intercellular canalization of adjacent endothelial processes (Figs. 12 and 13); by curvature of the EC (Lewis, 1925, 1931; Wakui, 1988). In general, it is accepted that the lumen of the capillary sprout is formed between the adjacent endothelial processes (Figs. 12,

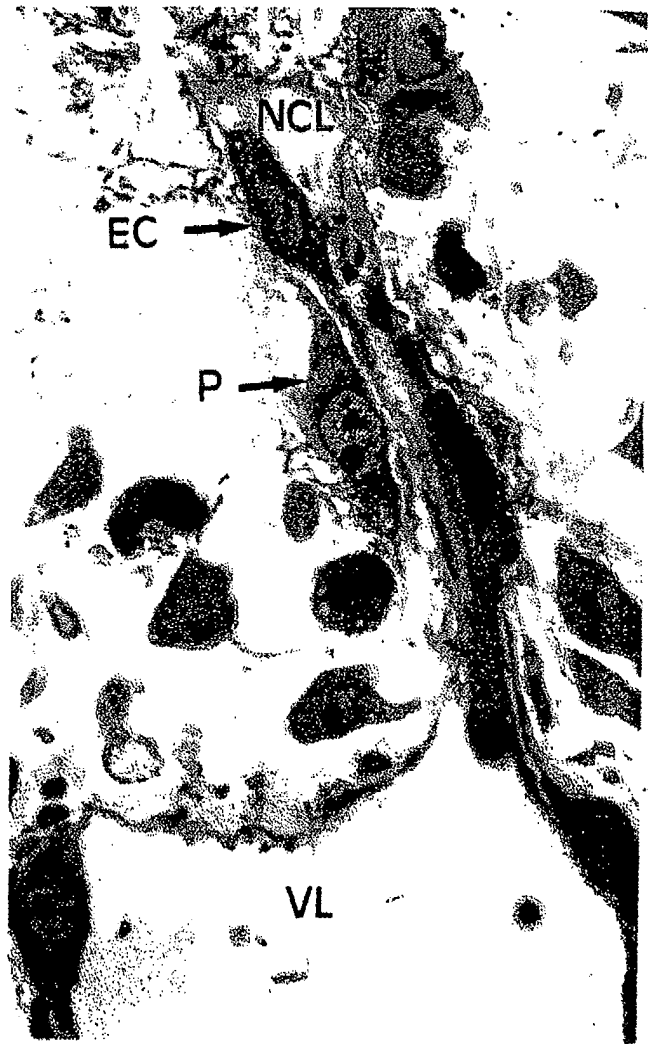


Fig. 12. Formation of the new capillary vessel lumen by intercellular canalization of adjacent endothelial cells. NCL: new capillary lumen; VL: lumen of the parent vessel; EC: endothelial cells; P: pericytes. Semithin section; toluidine blue. x 900

13),
conse
exact
conn
clarif
phase
and V
1988;
autho
sprou
1988)
an elc
1988;
vesse
with

Fig. 13.
P: peric

om pre

During
hannel
ve been
' vessel
cellular
tiguous
ion by
an and
; and b)
othelial
the EC
al, it is
formed
igs. 12.

(3), budding off the wall of the parent vessels and conserving their cellular polarity (Wakui, 1988). The exact moment in which the lumen of the new capillary connects with the parent vessel lumen has not been clarified. It may be from the beginning or in the early phases of the sprout formation (Yamagami, 1970; Bar and Wolff, 1972; Cavallo et al., 1973; Dvorak et al., 1988; Wakui, 1988; Paku and Paweletz, 1991). Some authors consider that the new lumen evolves while the sprouting EC are in the wall of the parent vessel (Wakui, 1988). In other words, the capillary sprout lumen may be an elongation of the parent vessel lumen (Wakui et al., 1988). Other authors are of the opinion that the new vessel lumen appears first in the sprout, merging later with the mother vessel (Ausprunk and Folkman, 1977;

Folkman, 1984). So that the lumen is developed by transversal division of the EC (Nicosia et al., 1982).

Pericytes in angiogenesis:

In the same way that EC are recognised as the principal cellular component of neovascularization, there is a controversy concerning the involvement of pericytes before and during the different phases of capillary sprouting (Burger and Klintworth, 1981). Regarding this problem, four aspects should be taken into account: a) the behaviour of the preformed microvasculature pericytes; b) the involvement of pericytes in the different stages of angiogenesis, including their incorporation to the newly formed capillaries; c) the origin of pericytes in

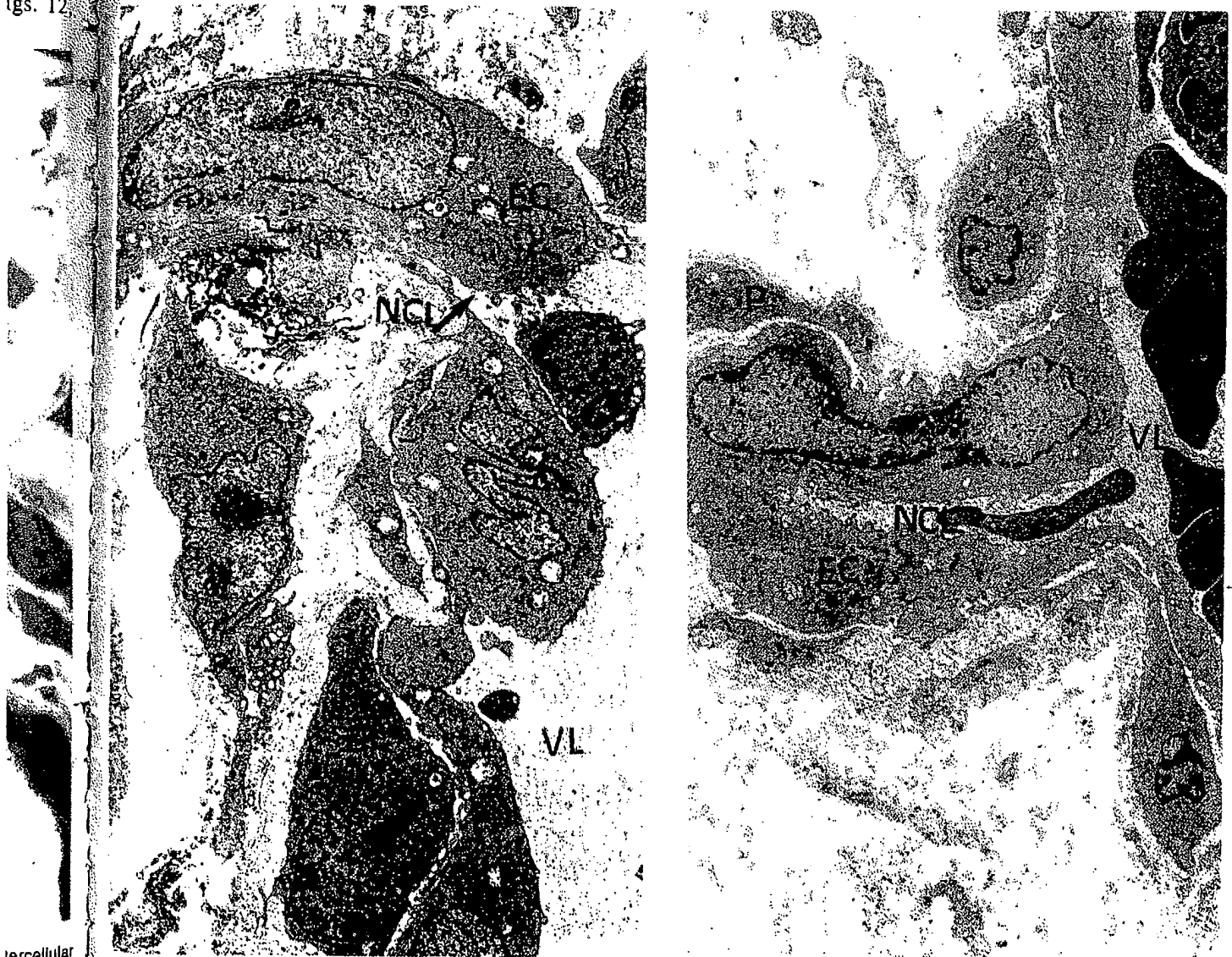


Fig. 13. Different stages of new capillary vessel lumen formation. NCL: new capillary lumen; VL: lumen of the parent vessel; EC: endothelial cells; P: pericytes. Ultrahin section; uranyl acetate and lead citrate. x 13,500

the newly formed vessels; and d) the role of pericytes in the regulation of EC proliferation during angiogenesis.

Information regarding modifications of pericytes at the level of the preformed (pre-existing) capillaries and postcapillary venules, from which the new blood vessels must develop, is scarce (Cavallo et al., 1973; McCracken et al., 1979; Burger and Klintworth, 1981; Diaz-Flores et al., 1992). The studies in preformed microvasculature pericytes reveal a sudden, brief and intense proliferation during the initial phase of angiogenesis (Diaz-Flores et al., 1992). Indeed, it has been pointed out that, after angiogenic stimulus, the pericytes undergo hypertrophy, with shortened processes, prominent nucleoli and dispersal of ribosomes into their free form (Fig. 7). Likewise, a decrease in contact surfaces between pericytes and endothelium, disruption and fragmentation of the pericyte basal lamina in some areas, and pericytic

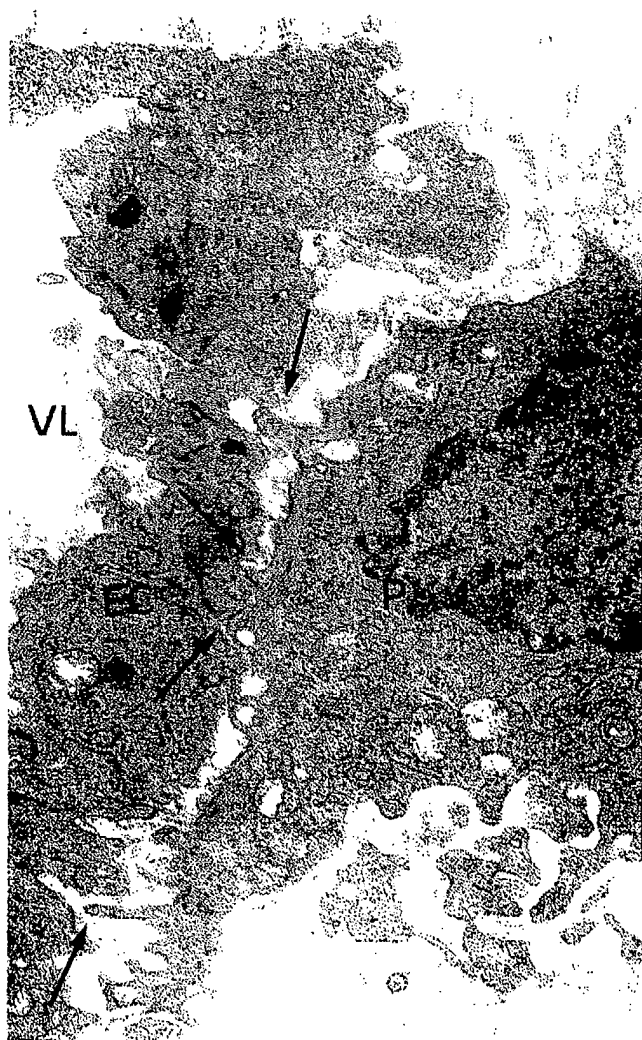


Fig. 14. The relationship between an endothelial cell and a pericyte in a growing capillary is shown. Cytoplasmic processes of the pericyte (P) and EC caving in on each other are observed (arrows). VL: vascular lumen. Ultrathin section; uranyl acetate and lead citrate. x 14,000

projections into the extravascular space have been observed (Fig. 6). In these conditions, many of the pericytes undergoing mitosis and autoradiographic studies show an increased DNA synthesis in pericytes and EC of the parent vessels (Fig. 10) (Schoefl, 1963; Cavallo et al., 1972, 1973; Sholley et al., 1977a,b; Burger and Klintworth, 1981; Diaz-Flores et al., 1992). In other words, the preformed microvasculature pericytes are substantially activated during post-natal angiogenesis, suggesting that they may contribute to the origin of new cells (Diaz-Flores and Dominguez, 1985; Diaz-Flores et al., 1988, 1989, 1990a,b, 1991a,b, 1992).

The second aspect is concerned with the question of pericyte involvement in the different stages of capillary sprouting. In the early stages of angiogenesis, the relationship between pericyte and endothelium remains unclear. Most of the authors are of the opinion that the involvement of capillaries with pericytes occurs at the end of the proliferative stage, following lumen formation (Folkman and Haudenschild, 1980; Folkman and Klagsbrun, 1987; D'Amore and Thompson, 1987; Paweletz and Knierim, 1989; Blood and Zetter, 1990). However, the number of pericytes increases when there is vascular proliferation (Schlingemann et al., 1990; Diaz-Flores et al. 1992) and EC may undergo mitosis when they are closely associated with pericytes (Cavallo et al., 1973; Sholley et al., 1977; Diaz-Flores et al., 1992a). The alternative possibility of an early recruitment of pericytes during angiogenesis has also been pointed out (Crocker et al., 1970; Inomata et al., 1971; Verhoeven and Buysens, 1988; Schlingemann et al., 1991; Nehls et al., 1992). Even the authors that consider an early incorporation of pericytes to the vascular sprouts, do not agree on the moment of occurrence. Thus, some authors describe the presence of pericytes surrounding the buds when the endothelial cells extend and/or migrate to form the endothelial sprout (Fig. 9), or when slit-lumen appear in them (Yamagami, 1970; Crocker et al., 1970; Inomata et al., 1971; Wakui, 1988). Other researchers believe that slit-like lumens develop when no pericytes are visible, pointing out that the first appearance of pericytes around the newly formed vessels bears no relationship to the development and organization of the basal lamina (Paku and Paweletz, 1991). The type of relationship between endothelial sprouts and nascent pericytes is also not totally clear. The fusion of pericytes with the endothelium at the point of active angiogenesis (Crocker et al., 1970; Inomata et al., 1971), and the presence of cytoplasmic processes of pericytes and EC caving in on each other (Wakui, 1988; Furusato et al., 1990) have been observed in the early stages of neovascularization (Fig. 14). Recently, nascent pericytes showing cellular processes advancing at the tips of endothelial sprouts have been described during angiogenesis. In this way, the gaps between opposing endothelial sprouts are bridged by pericytic processes, suggesting that pericytes may serve as guiding structures for EC outgrowth (Nehls et al., 1992). Therefore, capillary sprouting could

include (Nehls

Ano

pericyte

a primi

blood

1970),

1988)

sugges

fibrobl

Nehls e

pericyte

keratoc

capillar

keratoc

The fo

suppos

charact

contact

the peri

and c)

smooth

alpha a

been in

pericyt

new pe

Flores

muscle

precurs

between

includir

al., 19

Fujimot

as the g

cells (S

Final

a role in

1963; (

1987).

EC is t

presenc

may inl

and D' /

Char

basal la

Alth

been de

change

angioge

the par

intersti

Kalebic

microve

sulphate

present

1979; F

and Mo

been pericytes. Studies of EC of allo and other are genesis of new res et

include coordinated growth of both EC and pericytes (Nehls et al., 1992). Another open question is concerned with the origin of pericytes in the newly formed vessels. The hypotheses of a primitive origin from mesenchymal cells derived from blood monocytes around new vessels (Crocker et al., 1970), or from the EC of pre-existing vessels (Nakayasu, 1988) have been abandoned. Several observations suggest that they may evolve from perivascular fibroblasts (Nakayasu, 1988; Rhodin and Fujita, 1989; Nehls et al., 1992). In the corneal stroma, for example, pericytes of newly invading vessels may develop from keratocytes (Nakayasu, 1988). Likewise, formation of capillary-like tubes by vascular EC cocultivated with keratocytes has been described (Nakayasu et al., 1992). The following steps have been hypothesized in the supposition that fibroblasts acquire pericytic characteristics (Nehls et al., 1992): a) establishment of contacts between endothelial sprouts and fibroblasts; b) the periendothelial cells may start expression of desmin; and c) the periendothelial cells, or pericytes acquire smooth muscle-like features and begin to express SM alpha actin (Nehls and Drenckhahn, 1991). It has also been indicated that preformed pericytes of nonmuscular pericytic microvasculature contribute to the origin of new pericytes or other related cells (Cliff, 1976; Diaz-Flores et al., 1991a,b). Finally, the vascular smooth muscle cells have been considered as a possible precursor of pericytes due to the phenotypic similarities between both types of cells (Diaz-Flores et al., 1994), including certain smooth-muscle type proteins (Joyce et al., 1984a,b, 1985; Herman and D'Amore, 1985; Fujimoto and Singer, 1987; Skalli et al., 1989), as well as the gradual transition existing between both types of cells (Sims, 1986; Diaz-Flores et al., 1991b).

Finally, among the functions attributed to pericytes is a role in angiogenesis regulation (Kuwabara and Cogan, 1963; Crocker et al., 1970; Ordridge and D'Amore, 1987). The absence of pericytes at the tips of migrating EC is believed to stimulate EC mitosis, while their presence in the older regions of the growing capillaries may inhibit EC proliferation and migration (Ordridge and D'Amore, 1987; Sato and Rifkin, 1989).

Changes in extracellular matrix. Formation of a new basal lamina:

Although the basal lamina degradation has already been described, it will be considered along with the changes in the extracellular matrix. Indeed, during angiogenesis, local proteolysis of the basement lamina of the parent vessels and subsequent degradation of interstitial matrix are observed (Rifkin et al., 1982; Kalebic et al., 1983; Madri et al., 1983). In quiescent microvasculature, laminin, fibronectin, entactin, heparan sulphate proteoglycan and collagen types IV and V are present in the basement lamina (Kanwar and Farquhar, 1979; Foidart et al., 1980; Bender et al., 1981; Pepper and Montesano, 1990). Initially, the active EC secrete

matrix degrading enzymes, such as plasminogen activator and collagenase, causing fragmentation of the basement lamina (Folkman, 1982). Likewise, the components of the microvascular extracellular matrix, such as fibronectin, laminin and collagen Types I, III, IV and V, undergo dramatic changes (Nicosia and Madri, 1987), while EC undergo tube formation and extension (Folkman and Haudenschild, 1980; Folkman, 1986). In the initial stages of developing microvessels, fibronectin is the predominant component of the provisional matrix, making up a delicate fibrillary network. Fibrils of type V collagen, patchy amorphous deposits of laminin and Type IV collagen, and rare to absent fibrils of Type I and III collagen have also been described (Nicosia and Madri, 1987). Progressively, the deposits of fibronectin decrease, becoming discontinuous, while laminin and type IV collagen increase, accumulating and forming a continuous feltwork in the subendothelial space. In the late stages of angiogenesis, increased amounts of Types I and III collagen in the perivascular space are observed.

In some conditions, greatly thickened and/or multilayered basal lamina have been described in newly matured vessels (Szalay and Pappas 1970; Smelser and Ozanics, 1972). The possibility that this is due to repeated episodes of EC death and regrowth has been considered (Vracko and Benditt, 1970).

Capillary loop formation:

A short distance away from the parent vessel, the

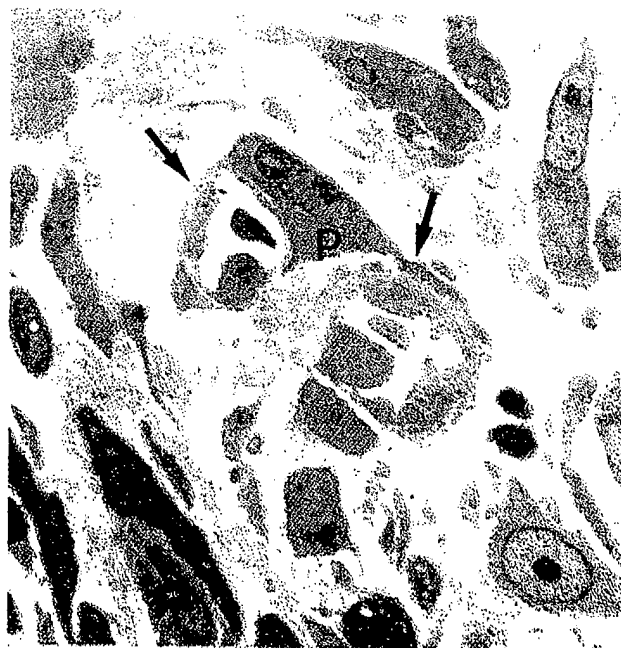


Fig. 15. The processes (arrows) of a pericyte (P) bridge between two capillary sprouts and they may serve as guiding structures for the fusion of sprouts into continuous capillary loops. Semithin section; toluidine blue. x 900

capillary sprouts, whose tips contain migrating EC, begin to branch and join other tips to form capillary loops. Other capillary sprouts then appear from these loops to form a plexus.

How capillary sprouts find each other to fuse into continuous capillary loops is an unsolved problem. It has been suggested that pericytic processes, which seem to bridge the gap between the leading edges of opposing endothelial sprouts, may serve as guiding structures for the outgrowth of EC (Nehls et al., 1992) (Fig. 15).

The capillary loop formation is well demonstrated during angiogenic reaction in the cornea, wherein secondary sprouts develop from the growing tips of initial sprouts, leading to a "brush border" morphology (Muthukkaruppan and Auerbach, 1979; Muthukkaruppan et al., 1982).

Early changes in the newly formed vessels. Persistence, involution and differentiation:

During angiogenesis, a substantial number of newly formed vessels regress (Meyer, 1852; Clark and Clark, 1939; Szalay and Pappas, 1970; Ausprunk et al., 1978; Latker and Kuwabara, 1981; Azmi and O'Shea, 1984; Latker et al., 1986; Spanel-Borowski and Mayerhofer, 1987). Indeed, immature vessels seem to require angiogenic stimuli to persist (Ausprunk et al., 1978), regressing when the stimuli are removed.

The sequence of events in the vessel regression is variable (Ausprunk et al., 1978; Azmi and O'Shea, 1984; Spanel-Borowski and Mayerhofer, 1987). Two main types of vascular regression may be considered (Ausprunk et al., 1978; Azmi and O'Shea, 1984). In the first type (Ausprunk et al., 1978), aggregation of



Fig. 16. Vascular regression during angiogenesis. Aggregation of platelets (P), stasis of blood (H) and degeneration of endothelial cells (EC) are observed. A, B and C. Semithin sections; toluidine blue x 900. D. Ultrathin section; uranyl acetate and lead citrate. x 13,500

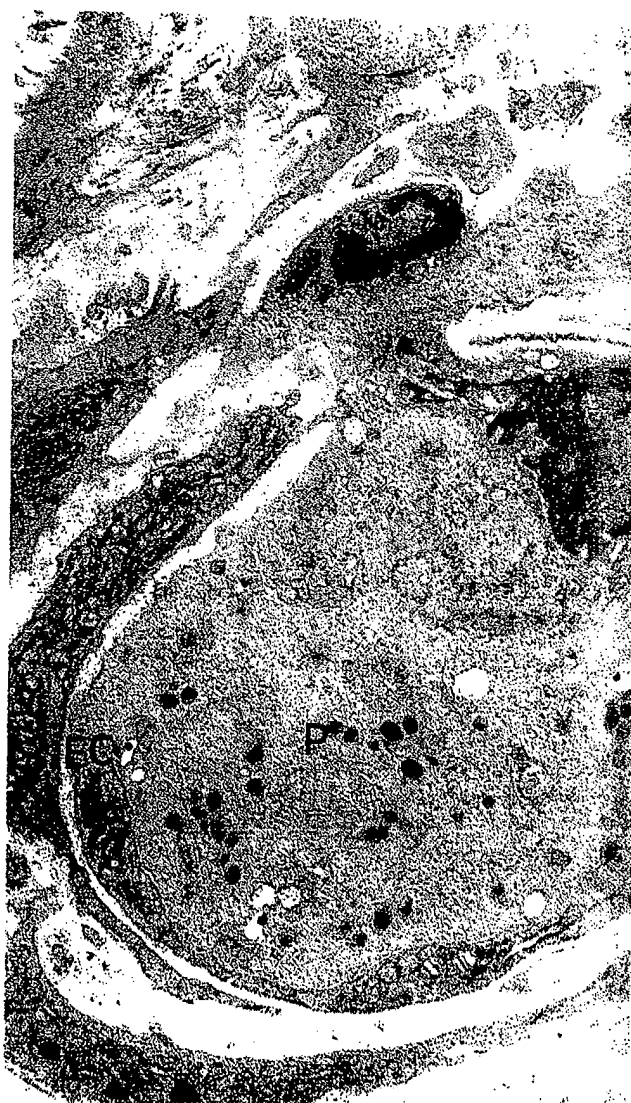


Fig. 17
are obs

sels. platelets, stasis of blood, vessel occlusion and degeneration of vessel wall cells are the most important findings (Fig. 16). The platelets accumulate and adhere to the endothelium of the regressing vessels. Furthermore, fibrin polymerization, stasis of blood and vessel occlusion by erythrocytes occur. Some of the erythrocytes also leak out into the interstitium. Due to the above, the presence of immature and involutive capillary aggregates, containing accumulated but frequently degranulated platelets, may behave as a "paracrine organ". On occasions, the EC become very thin or fenestrate. At other times the EC at the distal tips of the capillaries show organelle swelling, vacuolization, plasma membrane disruption and cytolysis followed by granular material deposition into the interstitium. Finally, mononuclear cells remove vascular debris.

In the second type of vascular regression (Azmi and O'Shea, 1984), the endothelial deletion is the greatest finding (Fig. 17). The following steps need to be

considered: a) protrusion of some individual EC into capillary lumen; b) formation of adherence junctions between the protruded ECs and other EC; c) nuclear and cytoplasmic condensation; d) cellular and nuclear lobation and fragmentation; e) disruption of cell organelles and loss of plasma membrane integrity and cytoplasmic density; and f) engulfment of degenerate cell fragments by viable mural EC.

Once some of the new vessels reach the source of the stimulus, there is a flow decrease in the less advanced vessels which then regress (Auerbach et al., 1991). At the same time, the vascular regression seems to occur first in the smallest distal capillary branches, probably because blood flow within them is sluggish (Ausprunk et al., 1978).

The regressive behaviour of blood vessels depends on the tissue. For example, in the cornea of some animals the newly formed vessel may disappear when the angiogenic stimulus is removed or ceases (Zauberman et



Fig. 17. Vascular regression during angiogenesis. Endothelial deletion and engulfment of degenerate cell fragments by viable mural endothelial cells are observed. EC: endothelial cells. Ultrathin sections; uranyl acetate and lead citrate, x 13,500

al., 1969; Ausprunk et al., 1978). On the contrary, newly formed blood vessels can persist in human corneas (Cogan, 1949). Likewise, in normally vascular tissues, regenerating blood vessels do not all regress (Jennings and Florey, 1970).

With regard to differentiation, the newly formed microvasculature may be influenced by the tissues within which it develops. Thus, although neo-vascularization in metastatic tumours arises from the blood vessels of the receptor site, it can acquire the morphological characteristics of the primary organ vessels in which the neoplasm originates. In other words, the new vessels can differentiate according to tissue specificity.

Capillary network formation and organization into larger microvessels:

The mechanism by which a functional circulation is established during post-natal angiogenesis is difficult to explain (Phillips et al., 1991). This mechanism may be undertaken in two different ways, both of which are probably associated (Burger et al., 1983; Philips et al., 1991; Diaz-Flores et al., 1994): 1) by remodelling the newly formed capillaries (Fig. 18) and their parent

vessels; and/or 2) by anastomoses of the new capillaries with pre-existing vessels of greater calibre in the venous and arterial sides of the circulation. The first possibility is based on the development of arteries and veins from capillary vessels, which would require a lateral EC proliferation and the presence of appropriate mural cells (pericytes and smooth muscle cells). Consistent with the second possibility for the venous side of the circulation, recent observations have demonstrated anastomoses of the microvessels originating from the small venules and capillaries with others arising from the veins (Diaz-Flores et al., 1994). As far as the arterial side of the circulation is concerned, a similar procedure does not seem likely, since new vessels have not been observed arising from arteries or arterioles. Nevertheless, an inverse process is possible, since ingrowing capillaries in the arterial wall have been described in several conditions (Diaz-Flores and Dominguez, 1985; Diaz-Flores et al., 1990a).

Peculiar forms of angiogenesis

Numerous forms of angiogenesis have been described. By way of examples, the vascularization of naturally avascular structures and tissue grafts, the



Fig. 18. Remodelling of the newly formed capillaries growing into a polyvinyl foam (F). L: vessel lumen; EC: endothelial cells; P: perivascular cells. Semithin section; toluidine blue. x 900

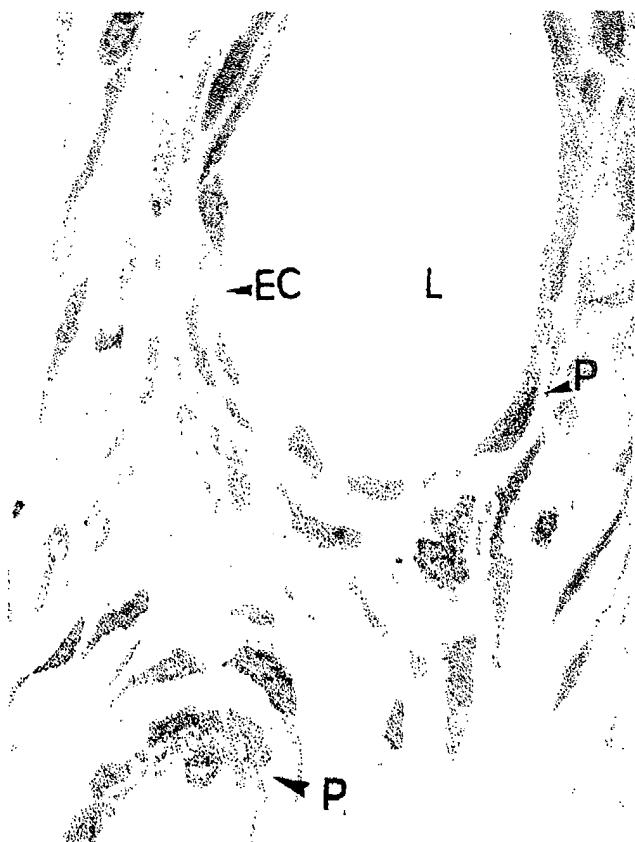


Fig. 19. A vessel (ar) section; u

growt
micro
of ne
vascul
will be

Vas

An
vessels
widely

The
has be
chick c
In thes
blood
tissues
ways.

vascul
the cor
memb
microv

Nec

Neo
models

growth intussusceptive in the post-natal pulmonary microcirculation, the angiogenesis during the formation of neovascular collateral vessels, the intimal vascularization of arteries and the tumour angiogenesis will be considered.

Vascularization of naturally avascular structures:

An example is the invasion of the cornea by blood vessels from the pericorneal plexus, an event that occurs widely in a variety of pathological states (Cogan, 1949).

The resistance of certain tissues to vascular invasion has been studied by means of explanting them onto chick chorioallantoic membrane (Eisenstein et al., 1973). In these conditions, the tissues, which normally have a blood supply, are rapidly invaded by host vessels. The tissues devoid of blood vessels behave in two opposing ways. Thus, hyalin cartilage is impenetrable by neovascularization, unless it is calcified, while the stroma of the cornea is readily penetrable, although Descemet's membrane forms a barrier against invasion by host microvessels.

Neovascularization of tissue grafts:

Neovascularization of tumours using syngenic in vivo models and of normal tissue grafts has been investigated

(Ausprunk and Folkman, 1977; Warren, 1979a; Paku and Paweletz, 1991). When re-vascularization of normal tissue grafts occurs, it is the result of the pre-existing graft vessels fusing with the host circulation (Folkman, 1976; Sasaki et al., 1991). Thus, newborn mouse cerebral cortex tissues transplanted into the third ventricle of rats show endothelial cells originating from both the host brain and the grafted mouse cerebral cortex, the latter expressing mouse-specific I ad antigen (Kohsaka et al., 1989). During neovascularization in the early stages of rat splenic autografts, it has been suggested that pre-existing sinus endothelial cells rearrange themselves after devascularization and reconstitute a characteristic complex structure that anastomoses with the invading capillaries from the connective tissue surrounding the graft (Sasaki et al., 1991).

In zones of focal necrosis or in related circumstances, such as grafts of autologous tissues, new EC sprouts from uninjured vessels can grow within the old basal lamina of necrotic vessels (Fig. 19), which provide a scaffold for the new vessels (Vracko and Benditt, 1970).

Growth intussusceptive in the postnatal pulmonary microcirculation:

An example of another proposed mechanism of microvascular growth is that termed as growth intussusceptive in the post-natal pulmonary microcirculation. The authors who present this hypothesis point out that the capillary bed grows by forming slender intravascular tissue pillars (Caduff et al., 1986; Burri and Tarek, 1990), in which interendothelial bridges are the contacts between opposite capillary walls. Subsequently, the contact areas are sealed off by building up inter-endothelial junctions, with a central perforation in the capillary layer. Successive perforations by cytoplasmic extensions of pericytes, myofibroblasts and interstitial fibres will transform the pillars into normal capillary meshes.

Angiogenesis in the formation of neovascular collateral vessels:

Although angiogenesis normally does not occur in most adult organs, an alternate route of blood supply may arise from preformed or neovascular collateral vessels in response to ischemic stimuli, such as that of an ischemic heart (Schaper and Vandesteene, 1967; de Brabander et al., 1973; Roth et al., 1990; Sasayama and Fujita, 1992; Carroll et al., 1993). This process of collateralization has been experimentally studied in chronic myocardial ischemia using dogs (Bloor and White, 1972; White et al., 1978; Unger et al., 1991) and pigs (Roth et al., 1987; White et al., 1992). In dogs, with an enormous potential for collateral growth, the collateral development is rapid and extensive, while in pigs, with a collateral angiogenesis more comparable to that found in human hearts, it is rapid but limited.

been
on of
s, the



Fig. 19. A new capillary growing within the old basal lamina of a necrotic vessel (arrows) is observed. EC: endothelial cell; P: pericyte. Ultrathin section; uranyl acetate and lead citrate. x 12,000

During collateralization, the underlying mechanisms in the genesis of newly formed vessels are likely to be similar to those involved in neovascular growth elsewhere (D'Amore and Thompson, 1987). Normal and ischemic myocardium contain heparin-binding angiogenic growth factor (D'Amore and Thompson, 1987; Thompson et al., 1988, 1989; Eghbali, 1989; Kardami and Frandrich, 1989; Quinkler et al., 1989; Sasaki et al., 1989; Casscells et al., 1990; Schmidt et al., 1991) and the release of mitogens in human (Kumar et al., 1983) and experimental animal (Galloway et al., 1984) hearts has been demonstrated. Likewise, heparin treatment accelerates coronary collateral development in experimental coronary artery occlusion (Carroll et al., 1993), increasing collateralization by potentiating the action of an ischemic-derived angiogenic factor (Fujita et al., 1988, 1991).

Intimal vascularization of arteries:

The microvessel invasion of the artery wall from the adventitia has been demonstrated in human pathology

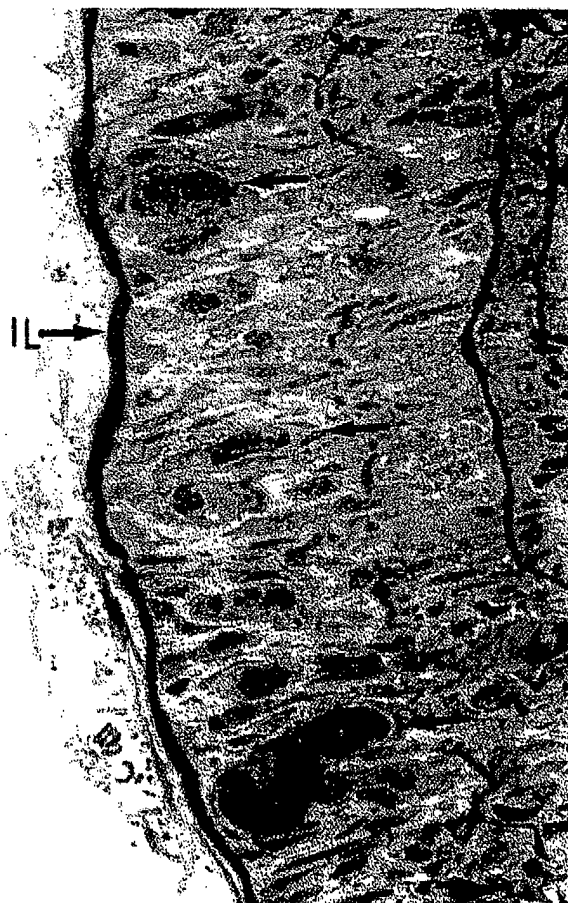


Fig. 20. Microvessel invasion of the artery wall. Several capillaries (arrows) are observed in the media of rat femoral artery after arterial wall injury. IL: Internal elastic lamina. Semithin section; toluidine blue. x 900

(Koester, 1876; Paterson, 1936, 1938; Barger et al., 1984; Eisenstein, 1991; Zhang et al., 1993) and in experimental conditions (Fig. 20) (Diaz-Flores et al., 1990a). The neovascularization of the arterial wall may play several important roles in the pathogenesis of intimal thickening and atherosclerosis, such as: a) supplying plasma components (albumin, fibrinogen ...) and thus nourishing the thickening wall during plaque growth (Koester, 1876; Le Compte, 1967; Groszek and Grundy, 1980; Zhang et al., 1993); b) causing intimal hemorrhages by rupturing (Paterson, 1938; Barger and Beeuwkes, 1990); and c) augmenting the process of arterial intimal thickening by supplying more cells. Indeed, in occluded arterial segments, it has been suggested that pericytes and EC of the ingrowing vessels from the arterial microcirculation are sources of myointimal cells at the intimal thickening and of endothelium at the luminal surface, respectively (Diaz-Flores and Dominguez, 1985; Diaz-Flores et al., 1990a, 1991b).

Tumour angiogenesis:

Tumour growth is accompanied by neovascularization (Ide et al., 1939; Warren and Shubik, 1966; Eddy and Cassarett, 1973; Yamaura and Sato, 1973; Tannock, 1968, 1970) (Fig. 21), since all solid tumours require stroma if they are to grow beyond 1 to 2 mm in size (about 10^6 cells) (Algire et al., 1945; Folkman et al., 1963; Folkman and Cotran, 1976; Reinhold and Van den Berg-Block, 1984; Folkman, 1985a,b,c, 1990). The tumour stroma is composed of new blood vessels, inflammatory cells and connective tissue (Dvorak, 1986). Thus, tumour growth beyond a few millimeters is dependent on angiogenesis (Folkman and Cotran, 1976; Folkman, 1971, 1972; Folkman and Haudenschild, 1980; Folkman and Klagsbrun, 1987), which is of great interest to cancer biology, metastasis, diagnosis and therapy. This newly formed microvasculature is induced by angiogenic substances (Algire et al., 1945; Folkman et al., 1971; Tuan et al., 1973; Klagsbrun and D'Amore, 1991) released by the tumour and by the inflammatory cells, predominantly macrophages (Polverini and Leibovich, 1984; Folkman and Klagsbrun, 1987). Furthermore, the decrease of angiogenesis inhibitors, such as thrombospondin, may occur when the cells undergo malignant transformation and become angiogenic (Rastinejad et al., 1989; Zajchowski et al., 1990).

The ingrowth of new blood vessels and other components of the stroma into the tumour cells closely resembles the granulation tissue of healing wounds (Dvorak et al., 1979a,b; Folkman, 1985a,b,c). Nevertheless, the organization of the microvasculature in the tumours is not so strict as in normal tissues. Tumours show phenotypic changes like dilated and irregular vessels (Warren, 1979a; Rofstad, 1984; Grunt et al., 1986), a high proliferation rate of the EC (Tannock, 1970; Cavallo et al., 1973; Denekamp, 1982) and increased permeability (Jain, 1985; Heuser and Miller,

1986).
Ac
import
neopla
in the
prevas
1973;
Sillman
1989a,
marked
differe

Fig. 21.
compose
EC: fenes
lead citrat

et al., 1986).

Acquisition of an angiogenic phenotype is an important fact in the transition from hyperplasia to neoplasia (Folkman et al., 1989a,b). Indeed, two phases in the development of tumours can be considered: prevascular; and vascular (Folkman and Hochberg, 1973; Gimbrone et al., 1974; Chodak et al., 1980; Sillman et al., 1981; Jensen et al., 1982; Folkman et al., 1989a,b). The study of the latter may be of interest as a marker for preneoplastic and neoplastic processes of different organs, such as cutaneous melanoma and



Fig. 21. The field shows a newly formed capillary in a melanoma composed of atypical cells containing melanosomes. (arrows). EC: fenestrated endothelial cell. Ultrathin section; uranyl acetate and lead citrate. x 14,500

carcinoma of the breast and bladder (Brem et al., 1977, 1978; Chodak et al., 1980; Sillman et al., 1981; Jensen et al., 1982; Srivastava et al., 1986, 1988). Transgenic mice expressing an oncogene in the pancreatic islet beta cells develop a progression from normal to hyperplasia and to neoplasia. Using these mice, it has been demonstrated that angiogenic activity and consequent neovascularization both precede tumour formation (Folkman et al., 1989a,b).

The intensity of vascularization in a tumour can predict the probability of metastasis (Liotta et al., 1974; Srivastava et al., 1986, 1988; Herlyn et al., 1987; Weidner et al., 1991), since there is a correlation between the density of tumour microvessels and the occurrence of metastases (Weidner et al., 1991). The fragmented basal lamina of growing vessels as well as collagenases and plasminogen activator secreted by the EC, make them more penetrable than mature vessels. Therefore, they give the tumour cells a greater chance to enter into the circulation (Liotta et al., 1974; Weidner et al., 1991).

Previously, we pointed out that neovascularization in metastatic tumours can acquire the morphological characteristics of the primary organ vessels in which the neoplasm originates. Likewise, the type of metastatic angiogenesis might be determined by the vascular peculiarities of the receptor organ. For example, liver metastases may be of sinusoidal or portal type. The first is formed by large convoluted vessels, devoid of immunohistochemically detectable basal lamina, while the portal type is characterized by numerous small vessels with basal lamina (Paku and Lapis, 1993).

Systems to analyze angiogenesis

In vivo

From the classical studies on new blood vessel morphogenesis and permeability (Sandison, 1928; Clark and Clark, 1935, 1939; Abell, 1946; Cogan, 1949; Friedman and Byers, 1962; Schoefl, 1963; Sugiura and Matsuda, 1969; Szalay and Pappas, 1970; Yamagami, 1970; Haar and Ackerman, 1971; Warren et al., 1972; McKinney and Panner, 1972; Cavallo et al., 1973), a large number of "in vivo" and "in vitro" systems have been used to understand the phenomenon of angiogenesis (Auerbach et al., 1991; Passaniti et al., 1992). The development of inert, biocompatible, slow-release polymer pellets, which permit a sustained release of angiogenic or antiangiogenic factors (Folkman et al., 1971; Langer and Folkman, 1976; Rhine et al., 1980; Hsieh et al., 1981; Murray et al., 1983), has been of interest for some of the experimental techniques. The "in vivo" experimental models include procedures where the angiogenic response in specific areas can be assessed, such as:

a) rabbit (Gimbrone et al., 1974; Brem and Folkman, 1975), rat (Fournier et al., 1981), mouse (Muthukkaruppan and Auerbach, 1979), or guinea pig

corneas, where neovascularization is produced by lesions, tumours (Gimbrone et al., 1974), or slow release polymers containing angiogenic substances implanted in "pockets" created in their central regions. Through this procedure, a linear quantitation of growing capillaries from the limbus is possible (Proia et al., 1988; Haynes et al., 1989; Culton et al., 1990). The cornea is an appropriate place to demonstrate neovascularization, since it is normally completely avascular and the new capillaries can be distinguished from parent vessels of the limbus (Ausprunk and Folkman, 1977; Henkind, 1978). Therefore, this procedure provides an "in vivo" avascular and transparent substratum in which neovascularization can be continuously monitored (Polverini et al., 1977a; Greenburg and Hunt, 1978). Several hypotheses have been proposed to explain the neovascularization in the cornea (Klintworth, 1991), such as liberation of angiogenic factors (Maurice et al., 1966; Eliason and Elliot, 1987; Vlodavsky et al., 1987; Baudouin et al., 1990; Soubrane et al., 1990), destruction of anti-angiogenic substances (Kuettner et al., 1974), inflammation (Fromer and Klintworth, 1975a,b; Polverini et al., 1977b; Klintworth, 1977; Epstein and Stulting, 1987), hypoxia and corneal swelling (Cogan, 1949). Corneal neovascularization could be the result of a local imbalance between angiogenic and anti-angiogenic factors (Kaminska and Niederkorn, 1993) originating from inflammatory cells that invade the cornea or from the cornea itself, together with loosening of the stroma during the inflammatory process.

b) The chorioallantoic membrane (Alfthan 1956; Sorgente et al., 1975; Auerbach, 1981; Folkman 1982; Shing et al., 1985; Fett et al., 1985; Olivo et al., 1992), or the yolk sac (Taylor and Folkman, 1982; Shing et al., 1985; Rosenbruch, 1989; Takigawa et al., 1990a,b) of the early chick embryo, which lacks a mature immune system allowing the growth of xenogeneic graft. Furthermore, by means of this second "in vivo" procedure a more rapid assay of either angiogenesis inhibitors or angiogenic factors is possible before selecting a few to be tested in the cornea (Folkman, 1985a,b,c). The main problem is the possibility of false positives produced by almost any irritant or wound, which may be avoided by incubating the embryos in Petri dishes (Auerbach et al., 1974).

c) Other "immunologically privileged" sites, such as the hamster cheek pouch (Warren and Shubik, 1966; Schreiber et al., 1986) and the anterior eye chamber (Greene, 1943), which allow the growth of allogeneic or xenogeneic grafts with a minor cell-mediated immune response (Greene, 1943; Folkman et al., 1989a,b; Weidner et al., 1991).

d) The subcutaneous air "pouch", dorsal air sac or "blister" method (Selye, 1953), the "sandwich" observation chamber, and the rabbit ear chamber.

e) The mesentery (Norrby et al., 1986; Williams et al., 1989; Norrby et al., 1990a,b).

f) The subcutaneous implants of polyurethane foam cylinders (Bishop et al., 1989, 1990) and the disc angio-

genesis assay. In the latter, discs of polyvinyl alcohol sponges, containing a slow-release polymer core and their flat sides sealed with millipore filters, are used (Fajardo et al., 1988). By means of this procedure the penetration of cells and the angiogenic response may be studied.

g) The implants of matrices, such as fibrin or gelatin, which act as angiogenesis initiators and enable the introduction of test substances (Dvorak et al., 1987).

h) Injection of cells or drugs entrapped in alginate (Plunkett and Hailey, 1990; Robertson et al., 1991).

In vitro systems

Angiogenesis or parts of this process have been investigated morphologically by different in vitro systems (Madri and Stenn, 1982; Furcht, 1986; Ingber et al., 1986; Dvorak et al., 1988; Heimark and Schwartz, 1988; Ingber and Folkman, 1988).

After the introduction of methods for EC culture (Jaffe et al., 1972; Gimbrone et al., 1973), EC isolated from both small and large vessels have been shown to be capable of forming random networks of capillary-like tubes "in vitro", when grown under appropriate culture conditions (Folkman and Haudenschild, 1980; Maciag et al., 1982; Kubota et al., 1982; Montesano and Orci, 1985; Pepper et al., 1990; Nguyen et al., 1992).

The "in vitro" systems with formation of capillary-like structures have been undertaken in cultured EC from various kinds of blood vessels (Jaffe et al., 1973; Folkman et al., 1979), such as human umbilical veins (Maciag et al., 1982), bovine aortas (Feder et al., 1983), bovine capillaries (Folkman and Haudenschild, 1980; Montesano et al., 1983) and rat capillaries (Madri et al., 1983; Sato et al., 1987). These procedures, along with the establishment of microvascular pericytes in culture enable the assessment of the following parameters (D'Amore and Thompson, 1987; Auerbach et al., 1991): 1) The recovery rate of a denuded surface in a confluent EC monolayer. In these conditions, the EC migration provides a quantitative assessment of the angiogenic response (Pepper et al., 1987, 1989, 1990). 2) EC locomotion and directionality (chemokinesis and chemotaxis, respectively) using different procedures (Auerbach et al., 1974, 1991; Obeso and Auerbach, 1984; Stokes et al., 1990; Taraboletti et al., 1990) such as phagokinetic track assay, in which individual cells move on a gold monolayer and phagocyte the colloidal particles, leaving migration tracks (Zetter, 1980, 1988; Rupnick et al., 1988; Weber et al., 1989). 3) The degree of EC proliferation in culture with test factors is determined by DNA synthesis, nuclear staining, DNA content, etc (Watt and Auerbach, 1986; Folkman and Ingber, 1987; Folkman and Klagsbrun, 1987; Ryan, 1988; Simionescu and Simionescu 1988, 1991). 4) Changes in EC function during angiogenesis, such as modulations in the production of cytokines (Shepro, 1988), protease release and fibrinolytic activity (Sueishi et al., 1989), and basal lamina synthesis. 5) Tube formation in relation to the

substrate (Maciag et al., 1982; Maciag, 1984; Madri and Pratt, 1986; Nicosia and Ottinetti, 1990). 6) The role of the pericytes in angiogenesis using coculture of both pericytes and EC (Orlidge and D'Amore, 1987, 1988). These possibilities for the study of EC function have been increased by utilizing 3-dimensional angiogenesis assays (Madri et al., 1988; Goto et al., 1993; Williams, 1993).

Angiogenesis control

Neovessel formation depends on several angiogenic stimuli to initiate and direct the proliferation and migration of ECs in the connective tissue (Folkman, 1982, 1985a,b,c; Furcht, 1986).

The most important factors in the control of angiogenesis seem to be the following: a) the angiogenic factors, capable of stimulating the EC migration and/or proliferation; b) the angiogenic inhibitors; c) the extracellular matrix modifications (role of the changes in extracellular matrix); and d) the intercellular interactions, for example, the "free edge effect" by absence of neighbouring EC (Schwartz et al., 1982). We shall consider the first three points.

Angiogenic factors

Angiogenesis is thought to be initiated by diffusible angiogenic factors, either after local activation of genes encoding them, or by release from their storages. In the past decade, several molecules have been shown to induce angiogenesis by acting in a direct or indirect way, including a variety of growth factors (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991; Folkman and Shing, 1992). An angiogenic factor is called "direct" when it is capable of inducing endothelial proliferation and/or migration "in vivo" and of stimulating endothelial cells "in vitro". When the "in vitro" action fails, or is inhibited, the angiogenic factor is considered to be "indirect" assuming that it mobilizes other direct factor(s) or cell(s) "in vivo". Likewise, the angiogenic factors may act mainly, or specifically on the EC, or on the contrary, may be pleiotropic, other cells also intervening, such as fibroblasts, smooth muscle cells, etc. Taking the above into account, the angiogenic factors may be chemotactic, mitogenic, chemotactic and mitogenic at the same time, or neither mitogenic nor chemotactic, but rather induce angiogenesis indirectly (Klagsbrun and Folkman, 1991). Among the direct angiogenic factors are the basic fibroblast growth factor (bFGF) (Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1987; Klagsbrun and Vlodavsky, 1988), acidic fibroblast growth factor (aFGF) (Folkman and Haudenschild, 1980; Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1987), vascular endothelial growth factor (VEGF) (Connolly et al., 1989; Keck et al., 1989; Leung et al., 1989) and platelet-derived endothelial growth factor (PD-EGF) (Thomas et al., 1985; Gospodarowicz et al., 1987; Miyazono et al.,

1987; Ishikawa et al., 1989; Ferrara et al., 1989; Leung et al., 1989). The indirect angiogenic growth factors cover a great number of substances, such as, Transforming growth factor alpha (TGF-alpha), Epidermal growth factor (EGF) (Schreiber et al., 1986), Transforming growth factor beta (TGF-beta) (Roberts et al., 1986), Tumour Necrosis factor alpha (TNF-alpha) (Frater-Schröder et al., 1987), Platelet derived growth factor (PDGF) (Sato et al., 1993), E series prostaglandin (Ziche et al., 1982), angiogenin (Fett et al., 1985; Hallahan et al., 1991), monobutyryl (Dobson et al., 1990), nicotinamide (Kull et al., 1987), adenosine, Okadaic acid (Oikawa et al., 1992), hydroxyecosatrienoic acid (Masferrer et al., 1991), some copper complexes (Ziche et al., 1982; Raju et al., 1984; Folkman and Klagsbrun, 1987; Brem et al., 1990), hyaluronic acid degradation products (West et al., 1985) and age-associated glycosylation end-products (Cuzzolino et al., 1990).

Some angiogenic molecules are present in adult tissues where angiogenesis is absent (Gullino, 1981); the possibility that the angiogenic response depends on local activation, or inactivation of these molecules has been considered (Ziche et al., 1992). For example, corneal tissue under angiogenic stimulation becomes richer in sialic acid (Ziche et al., 1989) and copper ions (Ziche et al., 1982; Raju et al., 1982). Likewise, changes in the ratio of different substances in local tissue composition may modify the angiogenic response. Thus, corneal neovascularization induced by angiogenic factors is stimulated, or repressed in the cornea by reduction or enhancement of the GM3/GD3 ratio of tissue gangliosides (Ziche et al., 1992).

Some of the known angiogenic factors have been completely purified and characterized, while others are still being studied. We shall consider some of the most important angiogenic factors.

Fibroblastic growth factors:

bFGF and aFGF, with a strong affinity for anionic glycosamin-glycan heparin (Baird and Ling, 1987, see role of heparin on angiogenesis) are able to stimulate both angiogenesis in vivo and vascular endothelial cell growth in vitro (Thomas et al., 1985; Montesano et al., 1986; Gospodarowicz et al., 1987). Beta fibroblast growth factor, a multifunctional peptide of 146 aminoacids (Esch et al., 1985) is one of the more potent angiogenic factors and has the ability to stimulate the following features (Gospodarowicz et al., 1987): a) migration of EC and SMC which change their morphology becoming bipolar (Terranova et al., 1985; Gospodarowicz et al., 1985; Montesano et al., 1986; Moscatelli et al., 1986; Tsuboi et al., 1990); b) stimulation of cell proliferation with mitogenesis of vascular endothelial cells, SMC and a wide variety of cell types (Schweigerer et al., 1987; Winkles et al., 1987; Sato and Rifkin, 1988); c) EC production of a urokinase-type plasminogen activator, procollagenase (Gross et al., 1983; Moscatelli et al., 1986;

Presta et al., 1986; Montesano et al., 1986; Gospodarowicz et al., 1987; Banda et al., 1987; Rifkin and Moscatelli, 1989), a membrane bound enzyme that degrades type IV collagen and prostomelysin; d) synthesis and deposition of extracellular matrix proteins, with effects on EC collagen, fibronectin and proteoglycan production (Tseung et al., 1982; Gospodarowicz, 1983; Gospodarowicz et al., 1987); for example, sprouting, or migrating EC switch their synthetic pattern to types I and III collagen (Madri et al., 1983); on the contrary, quiescent capillary EC synthesize type IV collagen; and e) differentiation of EC, influencing their phenotypic expression (Vlodavsky and Gospodarowicz, 1979; Vlodavsky et al., 1979; Greenberg et al., 1980).

bFGF is expressed during vascularization in the embryo (Risau et al., 1988) and in the adult, as that occurring in the ischemic heart (McNeil, 1980; Tomanek et al., 1989; Sasaki, 1989). Furthermore, in vivo administration of bFGF, by means of slow release polymers, induces intense angiogenesis (Baird and Bohlen, 1989). Growth of vasa-vasorum into the intima and media is observed in response to bFGF when infused onto the normal adventitia, or into the injured media of the rat carotid artery (Cuevas et al., 1991).

bFGF is mainly confined within the cells producing it (Folkman and Klagsbrun, 1987). Likewise, a high concentration of its inactive complex is stored within the extracellular matrix from which it can be released as a biologically active form by the actions of degradative enzymes (Folkman et al., 1988). When the EC are activated, they dissolve the extracellular matrix and bFGF is released. The latter bind with receptors on EC (Friesel et al., 1986) and vascular smooth muscle cells (Winkles et al., 1987) in which the binding of FGF to heparan sulphate is a prerequisite (Kiefer et al., 1990). Although bFGF seems to exert its effects on EC via a paracrine mode, it has been shown that endogenous bFGF produced by EC is important for EC migration and plasminogen activator production (Sato and Rifkin, 1988).

It has been pointed out that bFGF induces the production of prostaglandin E₂ by microvascular EC and that PGE₂ augments the production of cyclic AMP in these cells, stimulating their proliferation (Allison and Kowalski, 1989). Likewise, the angiogenic action of bFGF can be abolished by the systemic administration of drugs inhibiting prostaglandin synthesis (Fajardo et al., 1992).

Vascular endothelial growth factors (VEGF):

The VEGF (Ferrara and Henzel, 1989; Levy et al., 1989; Leung et al., 1989; Conn et al., 1990a,b; Ferrara et al., 1992), a secreted heparin-binding dimeric glycoprotein (Ferrara et al., 1991, 1992; Ferrara and Henzel, 1989), constitutes a family of angiogenic factors (Ferrara and Henzel, 1989; Leung et al., 1989; Conn et al., 1990a,b; Ferrara et al., 1992) also known as vascular

permeability factor (Senger et al., 1983, 1986; Leung et al., 1989; Connolly et al., 1989; Keck et al., 1989; Ferrara and Henzel, 1989; Conn et al., 1990a,b; Tisher et al., 1991; Ferrara et al., 1992), or vasculotropin (Plotet et al., 1989), in which their respective cDNAs have been cloned. At present, four different molecular species of VEGF are generated by alternative splicing mRNA (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆) (Leung et al., 1989; Houck et al., 1991; Tisher et al., 1991; Ferrara et al., 1992), with 121, 165, 189 and 206 aminoacids. The VEGF mRNA is expressed in vascularized tissues (Ferrara et al., 1992; Berse et al., 1992) and during capillary proliferation (Phillips et al., 1990; Ravindranath et al., 1992). Likewise, VEGFs promote angiogenesis in vivo (Leung et al., 1989; Plotet et al., 1989; Connolly, 1989) and they are EC-specific mitogens in vitro (Ferrara and Henzel, 1989; Ishikawa et al., 1989; Gospodarowicz et al., 1989; Conn et al., 1990a,b). VEGF binding sites have been identified in a majority of tissues and organs. The binding sites of VEGF in cultured EC have been described (Vaisman et al., 1990), where tyrosine kinase protein is its receptor. The above mentioned findings are consistent with the hypothesis that one of the physiological roles of VEGF is to promote neovascularization (Ferrara et al., 1992). In a study on spatial and temporal patterns of VEGF and VEGF receptor expression during natural angiogenic processes taking place within the female reproductive system, VEGF mRNA was found to be expressed in cells surrounding the expanding vasculature, while VEGF receptors were constitutively expressed in the endothelium, regardless of its proliferative status (Shweiki et al., 1993). Thus, VEGF may target the angiogenic response to specific areas. Furthermore, in this study, all cell types expressing VEGF were steroidogenic and/or steroid-responsive cells, which suggests that the VEGF expression is hormonally regulated (Shweiki et al., 1993).

It has been proposed that a tonic presence of VEGF may be required to maintain the differentiated state of microvessels and that suppressed expression of VEGF and/or its receptors may contribute to vessel regression (Ferrara et al., 1992).

Platelet-derived endothelial cell growth factor (PD-ECGF):

PD-ECGF, purified to homogeneity from human platelets (Miyazono et al., 1987), is a sequenced protein with a relative molecular mass of about 45,000 (Ishikawa et al., 1989), which stimulates EC growth and chemotaxis in vitro and angiogenesis in vivo (Ishikawa et al., 1989). Furthermore, PD-ECGF amplifies DNA synthesis activity of FGFs on EC (Folkman and Shing, 1992).

Tumour necrosis factor alpha (TNF):

The tumour necrosis factor alpha (TNF-alpha), a

cytol
of in
secre
in an
et al.
Ben-
indu
1987
inhib
al.,
micro
know
oppo
respo
TNF
ng),
et al.,
has b
macro
(Ben-
micro
Like
admi
intrav
extra
1992)

Pl
EC
Bowe
PDGF
vascu
al., 19
parac
has be
forma
myofi

Pro

An
are th
(Ben-
Auerb
1989)

trigge
chorio
part i
vascu
more,
triace
from v
clear t
have t
pathw
prost;
inflamm
phage:

cytokine mainly produced by macrophages and capable of inducing bFGF production in EC and of enhancing its secretion (Okamura et al., 1991), has a controversial role in angiogenesis (Frater-Schroder et al., 1987; Leibowich et al., 1987; Sato et al., 1987; Schweigerer et al., 1987; Ben-Ezra et al., 1990; Fajardo et al., 1992). TNF- α induces angiogenesis in vivo (Frater-Schroder et al., 1987; Leibowich et al., 1987), while it is a potent inhibitor of EC growth "in vitro" (Frater-Schroder et al., 1987; Schweigerer et al., 1987) as well as of microvascular sprouts (Sato et al., 1987). Currently, it is known that this controversy is due to dose-dependent opposing effects of TNF, which determine a bimodal response (Fajardo et al., 1992). In vivo, low doses of m-TNF (0.01 - 1 ng) induce angiogenesis (maximum at 0.1 ng), whereas high doses (1 and 5 μ g) inhibit it (Fajardo et al., 1992). Owing to their pro-inflammatory actions, it has been suggested that production of prostaglandins by macrophages may mediate the angiogenic effect of TNF (Ben-Ezra et al., 1990). In vitro, TNF is chemotactic for microvascular EC, although not for large vessel EC. Likewise, TNF- α action depends upon the administration route. When TNF- α is injected intravascularly it may cause necrosis and when injected extravascularly, it is angiogenic (Folkman and Shing, 1992).

Platelet-derived growth factor:

EC themselves may synthesize PDGF (DiCorleto and Bowen-Pope, 1983; Starksen et al., 1987). Functional PDGF receptors have been demonstrated on microvascular EC of some tissues (Beitz et al., 1991; Heldin et al., 1991) which suggests that PDGF is an autocrine or paracrine modulator during angiogenesis. Recently, it has been pointed out that PDGF may accelerate capillary formation by activating connective tissue cells, such as myofibroblasts, in the vicinity of EC (Sato et al., 1993).

Prostaglandins:

Among the prostanoid lipids with angiogenic activity are the prostaglandins of the E series (PGE1 and PGE2) (Ben-Ezra 1978a,b; Ziche et al., 1982; Form and Auerbach, 1983; Dobson et al., 1985; Ziche et al., 1985, 1989). They have been used as strong angiogenesis triggers in the rabbit cornea and in the chick embryo chorioallantoic membrane, and could play an important part in the cascade of events underlying the neovascularization process (Ziche et al., 1989). Furthermore, extravascular PGE1 and PGE2, associated with triacetate, are capable of inducing capillary sprouting from veins (Diaz-Flores et al., 1994). Although it is not clear how PGE1 and PGE2 induce capillary growth, they have been considered as acting in vivo by some indirect pathway (Folkman and Klagsbrun, 1987). Indeed, prostaglandin levels are elevated in wounds, inflammatory exudates, tumours and activated macrophages (Form and Auerbach, 1983). In these conditions,

the secretion of growth factors could be the result of PGE1 and PGE2 mobilizing and activating macrophages, or by some other unknown mechanisms (Folkman and Klagsbrun, 1987). Certain members of the prostanoid family, which are different from currently known prostaglandins, seem to be the factors of major angiogenic activity secreted by 3T3 adipocytes (Castellot et al., 1982; Dobson et al., 1985).

Angiogenin:

Angiogenin, isolated from human colon adenocarcinoma cell-conditioned medium (Fett et al., 1985), is a 14kD protein present in plasma at a relatively high concentration. In vivo, it induces intense angiogenesis (Fett et al., 1985) in the chicken chorioallantoic membrane (Fett et al., 1985) and in the rabbit cornea. Moreover, angiogenin has the following actions: a) it is a potent inhibitor of cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes (St. Clair et al., 1987); b) it activates EC phospholipase and phosphatase A2 (Bicknell and Vallee, 1988); and c) it stimulates EC prostacyclin secretion by activation of phospholipase A2 (Bicknell and Vallee, 1989).

Role of heparin on angiogenesis:

Heparin facilitates neovascularization (Ribatti et al., 1987; Ehrlich et al., 1988) by the following mechanisms: a) release of basic FGF from the extracellular matrix (Bashkin et al., 1989); b) protection of both a- and bFGF from inactivation (Gospodarowicz and Cheng, 1986; Herbert et al., 1988); c) increased aFGF activity (Herbert et al., 1988); and d) facilitation of the interaction between bFGF and EC (Bashkin et al., 1989). High-affinity receptors on the cell surface require heparin-like molecules to bind with FGF (Yayon et al., 1991) and VEGF (Gitay-Goren et al., 1992). This is probably accomplished by inducing a conformational change in the growth factors to allow them to interact with their receptors (Yayon et al., 1991; Gitay-Goren et al., 1992).

Heparin stimulates the secretion and activation of plasminogen activator (Lijnen and Collen, 1986; Falcone, 1989). Furthermore, its binding to plasminogen activator inhibitor-1 (Ehrlich et al., 1991), potentiates its neutralization with thrombin.

Angiogenesis antagonists:

Although the term "angiogenesis inhibitor" was not introduced until 1975 (Brem and Folkman, 1975), numerous factors were identified as inhibiting vessel formation. The study of these factors is of great interest since it may facilitate the development of antagonists for treatment of angiogenic diseases. For example, the process of angiogenesis during tumour growth is a potential target for tumour therapy (Folkman and Cotran, 1976; Denekamp, 1982; Schor and Schor, 1983). The angiogenic inhibitors include the cartilage-derived

inhibitor identified as tissue inhibitor of metalloproteinases (Carmichael et al., 1986; Moses et al., 1990), thrombospondin (Rastinejad et al., 1989; Good et al., 1990; Iruela-Arispe et al., 1991), protamine (Taylor and Folkman, 1982), platelet factor-4 (Hiti-Harper et al., 1978; Taylor and Folkman, 1982; Maione et al., 1990), interferon (Tsuruoka et al., 1988; White et al., 1989), angiostatic antibiotics (Ingber et al., 1990) deoxymannojirimycin (Nguyen et al., 1992), steroids (Crum et al., 1985) and angiogenesis inhibitors effective as a combination (Folkman et al., 1989a,b), synthetic peptides containing the amino acid sequence Arg-Gly-Asp, minocycline (Tamargo et al., 1991), difluoromethyl ornithine (Takigawa et al., 1990a,b), sulphatin chitin derivatives (Murata et al., 1991), DS-4152 sulphated polysaccharide from *Arthrobacter*, and bovine vitreous extract (Lutty et al., 1983).

Several studies have been undertaken to demonstrate that cartilage extracts can inhibit angiogenesis (Eisenstein et al., 1973, 1975; Brem and Folkman, 1975; Langer et al., 1976, 1980; Lee and Langer, 1983). The purification and characterization of neovascularization inhibitor from cartilage and from the conditioned media of chondrocytes have been recently reported (Moses et al., 1990). This cartilage derived protein negatively modulates the proliferation and migration of capillary EC. It is a powerful inhibitor of neovascularization "in vitro" and of embryonic and tumour-induced angiogenesis "in vivo". Furthermore, it is a collagenase and metalloproteinase inhibitor.

Thrombospondin, a high molecular weight multifunctional glycoprotein (Silverstein et al., 1986), stimulates different EC functions and modulates the activity of angiogenic factors (Taraboletti et al., 1990). Recently, it has been demonstrated that angiogenic macrophages produce not only positive but also negative angiogenesis regulators such as thrombospondin 1 (DiPietro and Polverini, 1993).

Protamine, an arginine-rich basic protein of 4,300 molecular weight, found in sperm with an affinity for heparin, is an angiogenesis inhibitor (Taylor and Folkman, 1982), although it is not used for the control of neovascularization because of its high toxicity (Folkman, 1985a,b,c).

Platelet factor-4, a platelet alpha-granule protein with high affinity for heparin, has an angiostatic effect which is probably due to specific inhibition of growth factor-stimulated EC proliferation. This angiostatic activity may be modulated through sulphated polysaccharides (Maione et al., 1990).

Interferons, regulatory proteins with potent biological activities, may be effective in inhibiting the proliferation of EC (Friesel et al., 1987; Sidky and Borden, 1987; Feldman et al., 1988). Interferons have been used as treatment for pathogenic neovascularization diseases such as Kaposi's sarcoma (Groopman et al., 1984; Rios et al., 1985; Real et al., 1986) and pulmonary hemangiomatosis (White et al., 1989).

A new class of angiostatic antibiotics, termed angio-

inhibins, have been identified among the fumagillin analogues to suppress the growth of a wide variety of tumours (Ingber et al., 1990). Among the angiostatics is the O-(Chloroacetylcarbamoyl) fumagillol or AGM-1470 which is 50 times more active than the fumagillin parent and has relatively few side-effects (Ingber et al., 1990).

Angiogenesis inhibitors, effective as a combination, include heparin with hydrocortisone (Folkman et al., 1989a,b), as well as beta-cyclodextrin-tetradecasulphate with hydrocortisone (Folkman et al., 1989a,b).

Deoxymannojirimycin, which prevents synthesis of hybrid and complex-type oligosaccharides, inhibits capillary tube formation in vitro (Nguyen et al., 1992).

Role of the changes in extracellular matrix

The different structural organization and composition of the extracellular matrix during vascular sprouting (see changes in extracellular matrix) is principally due to the fact that the EC secrete enzymes, which digest the pre-existing basement membrane (Kalebic et al., 1983; Montesano and Vassalli, 1985), and synthesize glycoproteins, proteoglycans and collagen (Ausprunk, 1982), which make up the extracellular matrix of new microvessels. Thus, the EC play an important role in the remodelling of the extracellular matrix which surrounds them during capillary development.

At the same time, changes in the composition of the extracellular matrix surrounding the EC may have important regulatory effects on the various stages of microvascular morphogenesis, modifying the organization, morphological features, function and behaviour of EC, such as cellular phenotype, migration and proliferation (Folkman and Haudenschild, 1980; Delves et al., 1982; Madri et al., 1983; Tseng et al., 1983; Montesano et al., 1983; Schor et al., 1983; Madri and Pratt, 1986; Nicosia and Madri, 1987). Thus, extracellular matrix proteins isolated from basal lamina facilitate differentiation of EC into tube-like structures, whereas interstitial collagens stimulate EC migration and proliferation (Madri and Williams, 1983; Montesano et al., 1983). These phenomena are associated with modifications in intracellular cytoskeletal components (Kocher and Madri, 1989). For example, EC plated onto basal lamina form tubelike structures after a short period in culture, but they neither proliferate nor migrate. EC plated onto a gel containing the extracellular matrix proteins, form capillary tubes within 24 h, but within 2-3 weeks when EC are plated onto a two-dimensional matrix, or onto plastic (Kubota et al., 1988). Likewise, EC embedded in a three-dimensional collagen matrix organize into a network of capillary-like tubes and acquire correct cellular polarization (Montesano et al., 1983). Therefore, the extracellular matrix influences the proliferation and migration of the vascular EC, and also their capacity to differentiate into capillary-like structures and to determine the correct cellular polarization (Montesano et al., 1983; Form et al., 1986;

Madri
Folkman
injur
Hem
D
com
(Forr
Leble
form.
Madi
W
com
vascu
This
basal
of act
Th
adhes
cellu
genes
major
of co
"in v
cell-n
conve
differ

Acknoi
Genera
92/006

Refer

Abell R
forr
cap
Allthar
hun
emt
34),
Algire
Vas
Vas
tran
Allison
prol
pha
rece
and
Altschul
and
Auerba
Lym
69-E
Auerba
proc
Biol.
Auerba

Madri and Pratt, 1986; Ingber et al., 1987; Ingber and Folkman, 1989). Finally, the rates of EC recovery after injury depend on the matrix components (Young and Herman, 1985).

During angiogenesis, different basal lamina components distribute around newly formed vessels (Form et al., 1986; Nicosia and Madri, 1987; Murray and Leblond, 1988), and these components can induce lumen formation as tube-like structures (Kubota et al., 1982; Maciag et al., 1982; Ingber and Folkman, 1988).

When the synthesis and the degradation of the components of the basal lamina is disturbed, neovascularization is inhibited (Ingber and Folkman, 1988). This supports the hypothesis that formation of a new basal lamina plays an important role in the development of active vessels.

The integrins, cell surface molecules which mediate adhesion to either neighbouring cells or to the extracellular matrix, are likely to play a key role in angiogenesis. Thus, anti-integrin antibodies directed to the major integrin receptors for the tube-permissive matrices of collagen and fibrin, enhance capillary tube formation "in vitro". This fact suggests that restriction of specific cell-matrix interactions can enhance capillary formation, converting EC from a proliferative phenotype towards differentiation.

Acknowledgements: This work was in part supported by Dirección General de Universidades e Investigación. Gobierno de Canarias. Grant 92/006

References

- Abell R.G. (1946). The permeability of blood capillary sprouts and newly formed blood capillaries as compared to that of older blood capillaries. *Am. J. Physiol.* 147, 237-241.
- Alfthan O.S. (1956). A comparative study of the growth of skin and human skin tumors on the chorioallantoic membrane of the embryonated chicken eggs. *Ann. Med. Exp. Biol. Fenn.* 9 (Suppl. 34), 1-78.
- Algire G.H., Chaikley H.W., Legallais F.Y. and Park H.D. (1945). Vascular reactions of normal and malignant tumors in vivo: I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J. Natl. Cancer Inst.* 6, 46-73.
- Allison A.C. and Kowalski J. (1989). Prostaglandins as transducers of proliferation signals in microvascular endothelial cells and the pharmacological control of angiogenesis. *Vascular endothelium, receptors and transduction mechanisms*. Catravas J.D., Gillis C.N., and Ryan U.S. (eds). Plenum Press. New York. pp 99-110.
- Altschul R. (1954). *Endothelium. Its Development, morphology, function and pathology*. MacMillan Co. New York. pp 28-31.
- Auerbach R. (1981). Angiogenesis-inducing factors: a review. In: *Lymphokines*. Vol IV. Pick E. (ed) Academic Press. New York. pp 69-88.
- Auerbach R., Kubal L., Knighton D. and Folkman J. (1974). A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* 41, 391-394.
- Auerbach R., Auerbach W. and Polakowski I. (1991). Assays for angiogenesis; a review. *Pharmacol. Ther.* 51, 1-11.
- Ausprunk D.H. (1982). Synthesis of glycoproteins by endothelial cells in embryonic blood vessels. *Dev. Biol.* 90, 79-90.
- Ausprunk D.H. and Folkman J. (1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* 14, 53-65.
- Ausprunk D.H., Faltermann K. and Folkman J. (1978). The sequence of events in the regression of corneal capillaries. *Lab. Invest.* 38, 284-294.
- Azmi T.I. and O'Shea J.D. (1984). Mechanism of deletion of endothelial cells during regression of the corpus luteum. *Lab. Invest.* 51, 206-217.
- Baird A. and Ling N. (1987). Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem. Biophys. Res. Commun.* 142, 428-435.
- Banda M.J., Herron G.S., Murphy G. and Werb Z. (1987). Regulation of metalloproteinase activity by microvascular endothelial cells. In: *Angiogenesis. Mechanisms and pathophysiology*. Rifkin D.B. and Klagsbrun M. (eds) Cold Spring Harbor Laboratory. New York. pp 101-109.
- Bar Th. and Wolff J.R. (1972). The formation of capillary basement membranes during internal vascularization of the rat's cerebral cortex. *Z. Zellforsch.* 133, 231-248.
- Barger A.C. and Beeuwkes R. (1990). Rupture of coronary vasa vasorum as trigger of acute myocardial infarction. *Am. J. Cardiol.* 66, 41G-43G.
- Barger A.C., Beeuwkes R., Lainey L.L. and Silverman K.J. (1984). Hypothesis: vasa vasorum and neovascularization of human coronary arteries: A possible role in the pathophysiology of atherosclerosis. *N. Engl. J. Med.* 310, 175-177.
- Bashkin P., Doctrow S., Klagsbrun M., Svahn C.M., Folkman J. and Vlodavsky I. (1989). Basic fibroblast growth factor binds to sub-endothelial extracellular matrix and is released by heparitinase and heparin-like molecules. *Biochemistry* 28, 1737-1743.
- Baudouin C., Fredj-Reygrobellet D., Caruelle J-P., Barritault D., Gastaud P. and Lapalus P. (1990). Acidic fibroblast growth factor distribution in normal human eye and possible implications in ocular pathogenesis. *Ophthalmic Res.* 22, 73-81.
- Bavisotto L.M., Schwartz S.M. and Heimark R.L. (1990). Modulation of Ca-dependent intercellular adhesion in bovine aortic and human umbilical vein endothelial cells by heparin-binding growth factors. *J. Cell. Physiol.* 143, 39-51.
- Bender B.L., Jaffe R., Carlin B. and Chung A.E. (1981). Immunolocalization of entactin, a sulfated basement membrane component, in rodent tissues, and comparison with GP-2 (laminin). *Am. J. Pathol.* 103, 419-426.
- Ben-Ezra D. (1978a). Neovascuogenic ability of prostaglandins, growth factors and synthetic chemoattractants. *Am. J. Ophthalmol.* 86, 455-461.
- Ben-Ezra D. (1978b). Neovascuogenesis. Triggering factors and possible mechanisms. *Surg. Ophthalmol.* 24, 167-176.
- Ben-Ezra D., Hemo I. and Maftzir G. (1990). In vivo angiogenesis activity of interleukins. *Arch. Ophthalmol.* 108, 573-576.
- Beitz J.G., Kim I., Calabresi P. and Frackelton A.R. Jr. (1991). Human microvascular endothelial cells express receptors for platelet derived growth factor. *Proc. Natl. Acad. Sci. USA* 88, 2021-2025.
- Berse B., Brown L.F., Van De Vatter L., Dvorak H.F. and Senger D.R. (1992). Vascular permeability factor (vascular endothelial growth

- factor) gene is differentially expressed in normal tissues, macrophages and tumors. *Mol. Biol. Cell* 3, 211-220.
- Bicknell R. and Vallee B.L. (1988). Angiogenin activates endothelial cell phospholipase C. *Proc. Natl. Acad. Sci. USA* 85, 5961-5965.
- Bicknell R. and Vallee B.L. (1989). Angiogenin stimulates endothelial cell prostacyclin secretion by activation of phospholipase A2. *Proc. Natl. Acad. Sci. USA* 86, 1573-1577.
- Birdwell C.R., Gospodarowicz D. and Nicholson G.L. (1978). Identification, localization, and role of fibronectin in cultured bovine endothelial cells. *Proc. Natl. Acad. Sci. USA* 75, 3273-3277.
- Bishop D.K., Jutila M.A., Sedmak D.D., Beattie M.S. and Orosz C.G. (1989). Lymphocyte entry into inflammatory tissues in vivo. Qualitative differences of high endothelial venule-like vessels in sponge matrix allografts vs isografts. *J. Immunol.* 142, 4219-4224.
- Bishop D.K., Sedmak D.D., Leppink D.M. and Orosz C.G. (1990). Vascular endothelial differentiation in sponge matrix allografts. *Hum. Immunol.* 28, 128-133.
- Blood C.H. and Zetter B.R. (1990) Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochem. Biophys. Acta* 1032, 89-118.
- Bloor C.M. and White F.C. (1972). Functional development of the coronary collateral circulation during coronary artery occlusion in the conscious dog. *Am. J. Physiol.* 67, 483-500.
- Brambell F.W.R. (1956). Ovarian changes. In: Marshall's Physiology of reproduction, 3rd ed. Vol. I. Parkes A.S. (ed). Lonsmans Green. London. pp 397-542.
- Brem H. and Folkman J.H. (1975). Inhibition of tumor angiogenesis mediated by cartilage. *J. Exp. Med.* 141, 427-438.
- Brem S.S., Gullino P.M. and Medina D. (1977). Angiogenesis: a marker for neoplastic transformation of mammary papillary hyperplasia. *Science* 195, 880-882.
- Brem S.S., Jensen H. and Gullino P.M. (1978). Angiogenesis as a marker of preneoplastic lesions of the human breast. *Cancer* 41, 239-244.
- Brem S.S., Zagzag D., Tsanacis A.-M.C., Gately S., Elkouby, M.P. and Brien S.E. (1990). Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by Penicillamine and the depletion of copper, an angiogenic cofactor. *Am. J. Pathol.* 137, 1121-1142.
- Brenk H.A.S.V. (1955). Studies in restorative growth processes in mammalian wound healing. *Br. J. Surg.* 43, 525-550.
- Bowersox J.C. and Sorgente N. (1982). Chemotaxis of aortic endothelial cells in response to fibronectin. *Cancer Res.* 42, 2547-2551.
- Burger P.C. and Klintworth G.K. (1981). Autoradiographic study of corneal neovascularization induced by chemical cautery. *Lab. Invest.* 45, 328-335.
- Burger P.C., Chandler D.B. and Klintworth G.K. (1983). Corneal neovascularization as studied by scanning electron microscopy of vascular casts. *Lab. Invest.* 48, 169-180.
- Burri P.H. and Tarek M.R. (1990). A novel mechanism of capillary growth in the rat pulmonary microcirculation. *Anat. Rec.* 228, 35-45.
- Caduff J.H., Fischer L.C. and Burri P.H. (1986). Scanning electron microscopic study of the developing microvasculature in the postnatal rat lung. *Anat. Rec.* 216, 154-164.
- Carmichael D.F., Sommer A., Thompson R.C., Anderson D.C., Smith C.G., Welgus H.G. and Stricklin G.P. (1986). Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. *Proc. Natl. Acad. Sci. USA* 83, 2407-2411.
- Carroll S.M., White F.C., Roth D.M. and Bloor C.M. (1993). Heparin accelerates coronary collateral development in a porcine model of coronary artery occlusion. *Circulation* 88, 198-207.
- Casscells W., Speir W., Sasse J., Klagsbrun M., Allen P., Lee M., Calvo B., Chiba M., Haggroth L, Folkman J. and Epstein S.E. (1990). Isolation, characterization and localization of heparin-binding growth factors in the heart. *J. Clin. Invest.* 85, 433-441.
- Castellot J.J. Jr., Karnovsky M.J. and Spiegelman B.M. (1982). Differentiation-dependent stimulation of neovascularization and endothelial cell chemotaxis by 3T3 adipocytes. *Proc. Natl. Acad. Sci. USA* 79, 5597-5601.
- Cavallo T., Sade R., Folkman J. and Cotran R.S. (1972). Tumor angiogenesis. Rapid induction of endothelial mitoses demonstrated by autoradiography. *J. Cell Biol.* 54, 408-420.
- Cavallo T., Sade R., Folkman J. and Cotran R.S. (1973). Ultrastructural autoradiographic studies of the early vasoproliferative response in tumor angiogenesis. *Am. J. Pathol.* 70, 345-362.
- Christianens G.C.M.L., Sixma J.J. and Haspels A.A. (1982) Hemostasis in menstrual endometrium: a review. *Obstet. Gynecol. Surv.* 37, 281-303.
- Chodak G.W., Haudenschild C., Gittes R.F. and Folkman J. (1980). Angiogenic activity as a marker of neoplastic and of preneoplastic lesions of the human bladder. *Ann. Surg.* 192, 762-771.
- Clark R.A.F. (1985). Cutaneous tissue repair: Basic biologic considerations. *I. J. Am. Acad. Dermatol.* 13, 701-725.
- Clark E.R. and Clark E.L. (1935). Observations on changes in blood vascular endothelium in the living animal. *Am. J. Anat.* 57, 385-438.
- Clark E.R. and Clark E.L. (1939). Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* 64, 251-301.
- Clark R.A.F., Lanigan J.M., Dellepella P., Manseau E., Dvorak H.F. and Colvin R.B. (1982a). Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J. Invest. Dermatol.* 70, 264-269.
- Clark R.A.F., Quinn J.H., Winn H.J., Lanigan J.H., Dellepella P. and Colvin R.B. (1982b). Fibronectin is produced by blood vessels in response to injury. *J. Exp. Med.* 156, 646-651.
- Cliff W.J. (1963). Observations on healing tissue: A combined light and electron microscopic investigation. *Philos. Trans. R. Soc. Lond. B. (Biol. Sci.)* 246, 305-325.
- Cliff W.J. (1965). Kinetics of wound healing in rabbit ear chambers: a time lapse cinemicroscopic study. *Q. J. Exp. Physiol.* 50, 79-89.
- Cliff W.J. (1976). The extra-endothelial cells of blood vessel wall. In: Blood vessels, biological structure and function. Vol. 6. Harrison R.J., McMin H.W. and Treherne K. (eds) Cambridge University Press. Cambridge. pp 68-72.
- Coffin J.D. and Poole T.J. (1988). Embryonic vascular development: Immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* 102, 735-748.
- Cogan D.G. (1949). Vascularization of the cornea: its experimental induction by small lesions and a new theory of its pathogenesis. *Arch. Ophthalmol.* 41, 406-416.
- Conn G., Soderman D.D., Schaffer M.-T., Wile M., Hatcher V. and Thomas K. (1990a). Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc. Natl. Acad. Sci. USA* 87, 1323-1327.
- Conn G., Bayne M., Soderman L., Kwok P.W., Sullivan K.A., Palisi T.M., Hope D.A. and Thomas K.A. (1990b). Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to

plate
2632
Connolly
Delf
Tum
and
Cozzolir
H., I
end
and
Crocker
wou
Crum R
inhib
fragr
Cuevas
resp
norm
Circ.
Culton M
G.K.
Inve
Davis M
Meta
D'Amori
gene
de Brab
chan
arter
Delvos
(198
Inve
Dernir F
(198
villi,
Denekar
to lai
Denekar
expe
Diaz-Flo
intim
165-
Diaz-Flo
Grow
peric
Diaz-Flo
Proli
Path
Diaz-Flo
role
intim
Diaz-Flo
(1991
relati
Diaz-Flo
Indur
graft
Diaz-Flo
(199

- factor) gene is differentially expressed in normal tissues, macrophages and tumors. *Mol. Biol. Cell* 3, 211-220.
- Bicknell R. and Vallee B.L. (1988). Angiogenin activates endothelial cell phospholipase C. *Proc. Natl. Acad. Sci. USA* 85, 5961-5965.
- Bicknell R. and Vallee B.L. (1989). Angiogenin stimulates endothelial cell prostacyclin secretion by activation of phospholipase A2. *Proc. Natl. Acad. Sci. USA* 86, 1573-1577.
- Birdwell C.R., Gospodarowicz D. and Nicholson G.L. (1978). Identification, localization, and role of fibronectin in cultured bovine endothelial cells. *Proc. Natl. Acad. Sci. USA* 75, 3273-3277.
- Bishop D.K., Jutila M.A., Sedmak D.D., Beattie M.S. and Orosz C.G. (1989). Lymphocyte entry into inflammatory tissues in vivo. Qualitative differences of high endothelial venule-like vessels in sponge matrix allografts vs isografts. *J. Immunol.* 142, 4219-4224.
- Bishop D.K., Sedmak D.D., Leppink D.M. and Orosz C.G. (1990). Vascular endothelial differentiation in sponge matrix allografts. *Hum. Immunol.* 28, 128-133.
- Blood C.H. and Zetter B.R. (1990) Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochem. Biophys. Acta* 1032, 89-118.
- Bloor C.M. and White F.C. (1972). Functional development of the coronary collateral circulation during coronary artery occlusion in the conscious dog. *Am. J. Physiol.* 67, 483-500.
- Brambell F.W.R. (1956). Ovarian changes. In: Marshall's Physiology of reproduction. 3rd ed. Vol. 1. Parkes A.S. (ed). Lonsmans Green. London. pp 397-542.
- Brem H. and Folkman J.H. (1975). Inhibition of tumor angiogenesis mediated by cartilage. *J. Exp. Med.* 141, 427-438.
- Brem S.S., Gullino P.M. and Medina D. (1977). Angiogenesis: a marker for neoplastic transformation of mammary papillary hyperplasia. *Science*. 195, 880-882.
- Brem S.S., Jensen H. and Gullino P.M. (1978). Angiogenesis as a marker of preneoplastic lesions of the human breast. *Cancer*. 41, 239-244.
- Brem S.S., Zagzag D., Tsanacis A.-M.C., Gately S., Elkouby, M.P. and Brien S.E. (1990). Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by Penicillamine and the depletion of copper, an angiogenic cofactor. *Am. J. Pathol.* 137, 1121-1142.
- Brenk H.A.S.V. (1955). Studies in restorative growth processes in mammalian wound healing. *Br. J. Surg.* 43, 525-550.
- Bowersox J.C. and Sorgente N. (1982). Chemotaxis of aortic endothelial cells in response to fibronectin. *Cancer Res.* 42, 2547-2551.
- Burger P.C. and Klintworth G.K. (1981). Autoradiographic study of corneal neovascularization induced by chemical cautery. *Lab. Invest.* 45, 328-335.
- Burger P.C., Chandler D.B. and Klintworth G.K. (1983). Corneal neovascularization as studied by scanning electron microscopy of vascular casts. *Lab. Invest.* 48, 169-180.
- Burri P.H. and Tarek M.R. (1990). A novel mechanism of capillary growth in the rat pulmonary microcirculation. *Anat. Rec.* 228, 35-45.
- Caduff J.H., Fischer L.C. and Burri P.H. (1986). Scanning electron microscopic study of the developing microvasculature in the postnatal rat lung. *Anat. Rec.* 216, 154-164.
- Carmichael D.F., Sommer A., Thompson R.C., Anderson D.C., Smith C.G., Welgus H.G. and Stricklin G.P. (1986). Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. *Proc. Natl. Acad. Sci. USA* 83, 2407-2411.
- Carroll S.M., White F.C., Roth D.M. and Bloor C.M. (1993). Heparin accelerates coronary collateral development in a porcine model of coronary artery occlusion. *Circulation* 88, 198-207.
- Casscells W., Speir W., Sasse J., Klagsbrun M., Allen P., Lee M., Calvo B., Chiba M., Haggroth L, Folkman J. and Epstein S.E. (1990). Isolation, characterization and localization of heparin-binding growth factors in the heart. *J. Clin. Invest.* 85, 433-441.
- Castellot J.J. Jr., Karnovsky M.J. and Spiegelman B.M. (1982). Differentiation-dependent stimulation of neovascularization and endothelial cell chemotaxis by 3T3 adipocytes. *Proc. Natl. Acad. Sci. USA* 79, 5597-5601.
- Cavallo T., Sade R., Folkman J. and Cotran R.S. (1972). Tumor angiogenesis. Rapid induction of endothelial mitoses demonstrated by autoradiography. *J. Cell Biol.* 54, 408-420.
- Cavallo T., Sade R., Folkman J. and Cotran R.S. (1973). Ultrastructural autoradiographic studies of the early vasoproliferative response in tumor angiogenesis. *Am. J. Pathol.* 70, 345-362.
- Christianens G.C.M.L., Sixma J.J. and Haspels A.A. (1982) Hemostasis in menstrual endometrium: a review. *Obstet. Gynecol. Surv.* 37, 281-303.
- Chodak G.W., Haudenschild C., Gittes R.F. and Folkman J. (1980). Angiogenic activity as a marker of neoplastic and of preneoplastic lesions of the human bladder. *Ann. Surg.* 192, 762-771.
- Clark R.A.F. (1985). Cutaneous tissue repair: Basic biologic considerations. *I. J. Am. Acad. Dermatol.* 13, 701-725.
- Clark E.R. and Clark E.L. (1935). Observations on changes in blood vascular endothelium in the living animal. *Am. J. Anat.* 57, 385-438.
- Clark E.R. and Clark E.L. (1939). Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* 64, 251-301.
- Clark R.A.F., Lanigan J.M., Dellepella P., Manseau E., Dvorak H.F. and Colvin R.B. (1982a). Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J. Invest. Dermatol.* 70, 264-269.
- Clark R.A.F., Quinn J.H., Winn H.J., Lanigan J.H., Dellepella P. and Colvin R.B. (1982b). Fibronectin is produced by blood vessels in response to injury. *J. Exp. Med.* 156, 646-651.
- Cliff W.J. (1963). Observations on healing tissue: A combined light and electron microscopic investigation. *Philos. Trans. R. Soc. Lond. B. (Biol. Sci.)* 246, 305-325.
- Cliff W.J. (1965). Kinetics of wound healing in rabbit ear chambers: a time lapse cinemicroscopic study. *Q. J. Exp. Physiol.* 50, 79-89.
- Cliff W.J. (1976). The extra-endothelial cells of blood vessel wall. In: Blood vessels, biological structure and function. Vol. 6. Harrison R.J., McMinn H.W. and Treherne K. (eds) Cambridge University Press. Cambridge. pp 68-72.
- Coffin J.D. and Poole T.J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* 102, 735-748.
- Cogan D.G. (1949). Vascularization of the cornea: its experimental induction by small lesions and a new theory of its pathogenesis. *Arch. Ophthalmol.* 41, 406-416.
- Conn G., Soderman D.D., Schaffer M.-T., Wile M., Hatcher V. and Thomas K. (1990a). Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc. Natl. Acad. Sci. USA* 87, 1323-1327.
- Conn G., Bayne M., Soderman L., Kwok P.W., Sullivan K.A., Pallis T.M., Hope D.A. and Thomas K.A. (1990b). Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to

plate
2632
Connolly
Delf
Tum
and
Cozzoli
H., I
end
and
Crocker
wou
Crum R
inhib
fragr
Cuevas
resp
norm
Circ.
Culton M
G.K.
Inver
Davis M
Meta
D'Amor
gene
de Brab
chan
arter
Delvos
(198
Inve
Demir F
(198
villi.
Denekar
to tar
Denekar
expe
Diaz-Flo
intim
165-
Diaz-Flo
Grov
perc
Diaz-Flo
Proli
Path
Diaz-Flo
role
intim
Diaz-Flo
(199
relate
Diaz-Flo
Indur
graft
Diaz-Flo
(199

- model of platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* 87, 2628-2632.
- I. Calko Connolly D.T., Heuvelman D.M., Nelson R., Olander J.V., Eppley B., Delfino J.J., Siegel R.N., Leingruber R.M. and Feder J. (1989). Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84, 1470-1478.
- (1982) Cuzzolino F., Torcia G., Ziche M., Ogawa S., Brett J., Koga S., Vlassara H., Nawroth P. and Stern D. (1990). Advanced glycosylation endproducts (AGEs) stimulate endothelial cell (EC) growth in vitro and in vivo. *Circulation* 82, 11136.
- ion and Crocker D.J., Murad T.M. and Geer J.C. (1970). Role of the pericyte in wound healing. An ultrastructural study. *Exp. Mol. Pathol.* 13, 51-65.
- ad. Sci. Tum R., Szabo S. and Folkman J. (1985). A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 230, 1375-1378.
- Tumor Cuebas P., Gonzalez A.N., Carcellar F. and Baird A. (1991). Vascular response to basic fibroblast growth factor when infused onto the normal adventitia or into the injured media of the rat carotid artery. *Circ. Res.* 69, 360-369.
- onstrated Gilton M., Chandler D.B., Proia A.D., Hickingbotham D. and Klintworth G.K. (1990). The effect of oxygen on corneal neovascularization. *Invest. Ophthalmol. Vis. Sci.* 31, 1277-1281.
- structural Davis M.D. (1988). Diabetic retinopathy: a clinical overview. *Diabetes/Metabol. Rev.* 4, 291-322.
- onse in D'Amore P.A. and Thompson R.W. (1987). Mechanisms of angiogenesis. *Annu. Rev. Physiol.* 49, 453-464.
- nostasis Brabander M., Scharper W. and Verheyen F. (1973). Regenerative changes in the porcine heart after gradual and chronic coronary artery occlusion. *Beitr. Pathol. Bd.* 149, 170-185.
- 37, 281- Delvos U., Gajdusek C., Sage H., Harker L.A. and Schwartz S.M. (1982). Interaction of vascular wall cells with collagen gels. *Lab. Invest.* 46, 61-72.
- (1980) Demir R., Kaufmann P., Castellucci M., Erbeni T. and Kotowski A. (1989). Fetal vasculogenesis and angiogenesis in human placental villi. *Acta Anat.* 136, 190-203.
- toplastic Denekamp J. (1982). Endothelial cell proliferation as a novel approach to targeting tumour therapy. *Br. J. Cancer* 45, 136-139.
- biologic Denekamp J. and Hobson B. (1982). Endothelial cell proliferation in experimental tumours. *Br. J. Cancer* 46, 711-720.
- in blood Diaz-Flores L. and Dominguez C. (1985). Relation between arterial intimal thickening and the vasa-vasorum. *Virchows Arch. (A)* 406, 165-177.
- 35-438 Diaz-Flores L., Rodriguez E., Gayoso M.J. and Gutierrez R. (1988). Growth of two types of cartilage after implantation of free autogenic perichondrial grafts. *Clin. Orthop.* 234, 267-279.
- i on the Diaz-Flores L., Martin A.I., Garcia R. and Gutierrez R. (1989). Proliferative fasciitis: ultrastructure and histogenesis. *J. Cutan. Pathol.* 16, 85-92.
- .nat. 64 Diaz-Flores L., Valladares F., Gutierrez R. and Varela H. (1990a). The role of the pericytes of the adventitial microcirculation in the arterial intimal thickening. *Histol. Histopath.* 5, 145-153.
- H.F. and Diaz-Flores L., Martin A.I., Garcia Montelongo R. and Gutierrez R. (1990b). Role of pericytes and endothelial cells in tissue repair and related pathological processes. *J. Cutan. Pathol.* 17, 191-192.
- ivisional Diaz-Flores L., Gutierrez R., Gonzalez P., and Varela H. (1991a). Inducible perivascular cells contribute to the neochondrogenesis in grafted perichondrium. *Anat. Rec.* 229, 1-8.
- alization Diaz-Flores L., Gutierrez R., Varela H., Rancel N. and Valladares F. (1991b). Microvascular pericytes: a review of their morphological and functional characteristics. *Histol. Histopath.* 6, 269-286.
- i P. and Diaz-Flores L., Gutierrez R., Lopez Alonso A., Gonzalez R. and Varela H. (1992). Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. *Clin. Orthop.* 275, 280-286.
- ssels in Diaz-Flores L., Gutierrez R., Valladares F., Varela H. and Perez M. (1994). Intense vascular sprouting from rat femoral vein induced by prostaglandins E1 and E2. *Anat. Rec.* 238, 68-76.
- light and DiCorleto P.E. and Bowen-Pope D.F. (1983). Cultured endothelial cells produce a platelet-derived growth factor-like protein. *Proc. Natl. Acad. Sci. USA* 80, 1919-1923.
- .ond. B DiPietro L.A. and Poverini P.J. (1993). Angiogenic macrophages produce the angiogenic inhibitor thrombospondin 1. *Am. J. Pathol.* 143, 678-684.
- nbres: a Dobson D.E., Castellot J.J. Jr., Spiegelman B.M. (1985). Angiogenesis stimulated by 3T3-Adipocytes is mediated by prostanoid lipids. *J. Cell Biol.* 101, 109a.
89. Dobson D.E., Kambe A., Block E., Dion T., Lu H., Castellot J.J. Jr and Spiegelman B.M. (1990). 1-Butyrylglycerol: a novel angiogenesis factor secreted by differentiating adipocytes. *Cell* 61, 223-230.
- wall. In Dvorak H.F. (1986). Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing. *New Engl. J. Med.* 315, 1650-1659.
- Harrison Dvorak H.F., Orenstein N.S. and Carvalho A.C. (1979a). Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J. Immunol.* 122, 166-174.
- iversity Dvorak H.F., Dvorak A.M., Manseau E.J., Wiberg L. and Churchill W.H. (1979b). Fibrin gel investment associated with line 1 and line 10 solid tumor growth, angiogenesis, and fibroplasia in guinea pigs: role of cellular immunity, myofibroblasts, microvascular damage, and infarction in line 1 tumor regression. *JNCI* 62, 1459-1472.
- opment Dvorak H.F., Harvey V.S., Estrella P., Brown L.F., McDonach J. and Dvorak A.M. (1987). Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. *Lab. Invest.* 57, 673-686.
- sequent Eddy H.A. and Cassarett G.W. (1973). Development of the vascular system in the hamster malignant neurilemmoma. *Microvasc. res.* 6, 63-82.
- mbryos Edwards R.G. (1980) Conception in the human female. Academic Press. London.
- rimental Eghbali M. (1989). Cellular origin and distribution of transforming growth factor-beta 1 in the normal rat myocardium. *Cell Tissue Res.* 256, 553-558.
- genesis Ehrlich H.P., Jung W.K., Costa D.E. and Rajaratnam J.B. (1988). Effects of heparin on vascularization of artificial skin grafting. *Exp. Mol. Pathol.* 48, 244-251.
- V. and Ehrlich H.J., Keijer J., Preissner K.T., Gebbink R.K. and Pannekoek H. (1991). Functional interaction of plasminogen activator inhibitor type 1 (PAI-1) and heparin. *Biochemistry* 30, 1021-1028.
- ar. endo Eisenstein R. (1991). Angiogenesis in arteries: Review. *Pharmacol. Ther.* 49, 1-19.
- oc. Natl. Eisenstein R., Sorgente N., Soble L.W., Miller A. and Kuettner K.E. (1973). The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.* 73, 765-774.
- list T.M. (1973). The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.* 73, 765-774.
- d cDNA (1973). The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.* 73, 765-774.
- gous to (1973). The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.* 73, 765-774.

- Eisenstein R., Kuettner K.E., Neopolitan C., Soble L.W. and Sorgenfe N. (1975). The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. *Am. J. Pathol.* 81, 337-348.
- Eklom P., Sariola H., Karkinen M. and Saxen L. (1982). The origin of the glomerular endothelium. *Cell Diff.* 11, 35-39.
- Eliason J.A. and Elliott J.P. (1987). Proliferation of vascular endothelial cells stimulated in vitro by corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 28, 1963-1969.
- Epstein R.J. and Stulting R.D. (1987). Corneal neovascularization induced by stimulated lymphocytes in inbred mice. *Invest. Ophthalmol. Vis. Sci.* 28, 1505-1513.
- Esch F., Baird A., Ling N., Ueno N., Hill F., Denoroy L., Kleppe R., Gospodarowicz D., Bohlen P. and Guillemin R. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine acidic FGF. *Proc. Natl. Acad. Sci. USA* 82, 6507-6511.
- Fajardo L.F., Kowalski J., Kwan H.H., Prionas S.D. and Allison A.C. (1988). The disc angiogenesis system. *Lab. Invest.* 58, 718-724.
- Fajardo L.F., Kwan H.H., Kowalski J., Prionas S.D. and Allison A.C. (1992). Dual role of tumor necrosis factor alpha in angiogenesis. *Am. J. Pathol.* 140, 539-544.
- Falcone D.J. (1989). Heparin stimulation of plasminogen activator secretion by macrophage-like cell line RAW264.7: role of the scavenger receptor. *J. Cell. Physiol.* 140, 219-226.
- Feder J., Marasa J.C. and Olander J.V. (1983). The formation of capillary-like tubes by calf aortic endothelial cells grown in vitro. *J. Cell. Physiol.* 116, 1-6.
- Feinberg R.N., Sherer, G.K. and Auerbach R. (1991). The development of the vascular system. S. Karger, Basel.
- Feldman D., Goldstein A.L., Cox D.C. and Grimley P.M. (1988). Cultured human endothelial cells treated with recombinant leukocyte A interferon: tubuloreticular inclusion formation, antiproliferative effect, and 2',5'oligoadenylate synthetase induction. *Lab. Invest.* 58, 584-589.
- Ferrara N. and Henzel W.J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 851-859.
- Ferrara N., Leung D.W. and Phillips H.S. (1991). Molecular characterization and distribution of vascular endothelial growth factor. In: *Neuroendocrine perspectives*. Muller E.E. and McLeod R.B. (eds). Springer Verlag, New York. 9, 127-161.
- Ferrara N., Houck K., Jakeman L. and Leung D.W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endoc. Rev.* 13, 18-32.
- Fett J.W., Strydom D.J., Lobb R.R., Alderman E.M., Bethune J.L., Riordan J.F. and Vallee B.L. (1985). Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry* 24, 5480-5486.
- Findlay J.K. (1986). Angiogenesis in reproductive tissues. *J. Endocrinol.* 111, 357-366.
- Foidart J.M., Bere E.W. Jr., Year M., Rennard S.I., Gullino M., Martin G.R. and Katz S.I. (1980). Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. *Lab. Invest.* 42, 336-342.
- Foley M.E., Griffin B.D., Zuzel M., Aparicio S.R., Bradbury K., Bird C.C., Clayton J.K., Jenkins D.M., Scott J.S., Rajah C.M. and McNicol G.P. (1978). Heparin-like activity in uterine fluid. *Br. Med. J.* ii, 322-324.
- Folkman J. (1971). Tumor angiogenesis. Therapeutic Implications. *New Engl. J. Med.* 285, 1182-1186.
- Folkman J. (1972). Anti-angiogenesis: new concept for therapy of solid tumors. *Ann. Surg.* 175, 409-416.
- Folkman J. (1975). Tumor angiogenesis. *Adv. Cancer Res.* 43, 175-203.
- Folkman J. (1976). The vascularization of tumors. *Sci. Am.* 234, 58-73.
- Folkman J. (1982). Angiogenesis: initiation and control. *Ann. N.Y. Acad. Sci.* 401, 212-227.
- Folkman J. (1984). What is the role of endothelial cells in angiogenesis. *Lab. Invest.* 51, 601-602.
- Folkman J. (1985a). Toward an understanding of angiogenesis search and discovery. *Pers. Biol. Med.* 29, 10-36.
- Folkman J. (1985b). Tumor angiogenesis. *Adv. Cancer Res.* 43, 175-203.
- Folkman J. (1985c). Angiogenesis and its inhibitors. In: *Important Advances in Oncology*. De Vita V.T. Jr, Hellman S.A. and Rosenberg S.A. (eds) Lippincott, Philadelphia. pp 42-62.
- Folkman J. (1986). How is blood vessel growth regulated in normal and neoplastic tissue. *Cancer Res.* 46, 467-473.
- Folkman J. (1989). Successful treatment of an angiogenic disease. *N. Engl. J. Med.* 320, 1211-1212.
- Folkman J. (1990). What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* 82, 4-6.
- Folkman J. and Cotran R. (1976). Relation of vascular proliferation to tumor growth. *Int. Rev. Exp. Pathol.* 16, 207-248.
- Folkman J. and Haudenschild C.C. (1980). Angiogenesis in vitro. *Nature* 288, 551-556.
- Folkman J. and Hochberg M. (1973). Self-regulation of growth in three dimensions. *J. Exp. Med.* 138, 745-753.
- Folkman J. and Ingber D.E. (1987). Angiostatic steroids. Method of discovery and mechanism of action. *Ann. Surg.* 206, 374-383.
- Folkman J. and Klagsbrun M. (1987). Angiogenic factors. *Science* 235, 442-447.
- Folkman J. and Shing Y. (1992). Angiogenesis. *J. Biol. Chem.* 267, 10931-10934.
- Folkman J., Long D.M. and Becker F.F. (1963). Growth and metastasis tumor in organ culture. *Cancer* 18, 453-467.
- Folkman J., Merler E., Abernathy C. and Williams G. (1971). Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* 133, 275-288.
- Folkman J., Haudenschild C.C. and Zetter B.R. (1979). Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA* 76, 5217-5221.
- Folkman J., Klagsbrun M., Sasse J., Wadzinski M.G., Ingber D. and Vlodavsky I. (1988). A heparin-binding angiogenic protein-basic fibroblast growth factor is stored within basement membrane. *Am. J. Pathol.* 130, 393-400.
- Folkman J., Watson K., Ingber D. and Hanahan D. (1989a). Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* 339, 58-62.
- Folkman J., Weisz P.B., Jouille M.M., Li W.M. and Ewing W.R. (1989b). Control of angiogenesis with synthetic heparin substitutes. *Science* 243, 1490-1493.
- Form D.M. and Auerbach R. (1983). PGE2 and angiogenesis. *Proc. Soc. Exp. Biol. Med.* 172, 214-218.
- Form D.M., Pratt B.M. and Madri J.A. (1986). Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components. *Lab. Invest.* 55, 521-530.
- Forsman A.D. and McCormack J.T. (1992). Microcorrosion casts of hamster luteal and follicular vasculature throughout the estrous

- cycle. *Anat. Rec.* 233, 515-520.
- of solid Fournier G.A., Iutty G.A., Watt S., Fenselau A. and Patz A. (1981). A corneal micropocket assay for angiogenesis in the rat eye. *Invest. Ophthalmol. Vis. Sci.* 21, 351-354.
- 75-203 Frater-Schroder M., Risau W., Hallmann, R., Gautschi P. and Bohlen P. (1987). Tumor necrosis factor type-alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc. Natl. Acad. Sci. USA* 84, 5277-5281.
- 58-73. Frederick J.L., Shimanuki T. and di Zerega G.S. (1984) Initiation of angiogenesis by human follicular fluid. *Science* 224, 389-390.
- (Acad. Freemont A.J. and Ford W. (1985). Functional and morphological changes in post-capillary venules in relation to lymphocytic infiltration into BCG-induced granulomata in rat skin. *J. Pathol.* 147, 1-12.
- genesis Friedman M. and Byers S.O. (1962). Excess lipid leakage: a property of very young vascular endothelium. *Br. J. Exp. Pathol.* 43, 363-372.
- search Friesel R., Burgess W.H., Mehlman T. and Maciag T. (1986). The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. *J. Biol. Chem.* 261, 7581-7584.
- 13, 175 Friesel R., Komoriya A. and Maciag T. (1987). Inhibition of endothelial cell proliferation by gamma-interferon. *J. Cell Biol.* 104, 689-696.
- portant Fromer C.H. and Klintworth G.K. (1975a). An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. I. Comparison of experimental models of corneal vascularization. *Am. J. Pathol.* 79, 537-554.
- A. and Fromer C.H. and Klintworth G.K. (1975b). An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. II. Studies on the effect of leukocyte elimination on corneal vascularization. *Am. J. Pathol.* 81, 531-544.
- nal and Fromer C.H. and Klintworth G.K. (1976). An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. III. Studies related to the vasoproliferative capability of polymorphonuclear leukocytes and lymphocytes. *Am. J. Pathol.* 82, 157-170.
- case. N. Fujimoto T. and Singer S.J. (1987). Immunocytochemical studies of desmin and vimentin in pericapillary cells of chicken. *J. Histochem. Cytochem.* 35, 1105-1115.
- genesis Fujita M., Sasayama S., Asanoi H., Nakajima H., Sakai O. and Ohno A. (1988). Improvement of treadmill capacity and collateral circulation as a result of exercise with heparin pretreatment in patients with effort angina. *Circulation* 77, 1022-1029.
- ation to Fujita M., Yamanishi K., Hirai T., Ohno A., Miwa K. and Sasayama S. (1991). Comparative effect of heparin treatment with and without strenuous exercise on treadmill capacity in patients with stable effort angina. *Am. Heart J.* 122, 453-457.
- . Nature Furcht L.T. (1986). Critical factors controlling angiogenesis: Cell products, cell matrix and growth factors. *Lab. Invest.* 55, 505-509.
- in three Furusato M., Fukunaga M., Kikuchi Y., Yokota S., Joh K., Aizawa S. and Ishikawa E. (1984). Two- and Three dimensional ultrastructural observation of angiogenesis in juvenile hemangioma. *Virchows Arch. (Cell Pathol.)* 46, 229-237.
- ethod of Furusato M., Shimoda T., Yokota K., Joh K., Miyazaki H., Inomata I., Takaki K., Aizawa S. and Ishikawa E. (1985). Angiogenesis of juvenile hemangioma. In: *Microcirculation annual 1985*. Tsuchiya M., Asano M. and Mishima Y. (eds). Elsevier, Amsterdam, pp 101-107.
- i. Furusato M., Wakui S., Suzuki M., Takagi K., Hori M., Asari M., Kano Y. and Ushigome S. (1990). Three dimensional ultrastructural distribution of cytoplasmic interdigitation between endothelium and pericyte of capillary in human granulation tissue by serial section reconstruction method. *J. Electron. Microsc.* Tokyo 39, 86-91.
- 235 Galloway A.C., Pelletier R. and D'Amore P.A. (1984). Do ischemic hearts stimulate endothelial cell growth? *Surgery* 96, 435-438.
- n. 267 Garbett P.K. and Gibbins J.R. (1987). Experimental neovascularization in vivo: the early changes in a stable adult vasculature responding to angiogenic stimulation by a syngeneic neoplasm. *Br. J. Exp. Pathol.* 68, 625-635.
- tastasis Gimbrone M.A. Jr., Cotran R.S. and Folkman J. (1973). Endothelial regeneration: studies with human endothelial cells in culture. *Ser. Haematol.* 6, 453-455.
- lation of Gimbrone M.A. Jr., Cotran R.S., Leapman S.B. and Folkman J. (1974). Tumor growth and neovascularization: An experimental model using the rabbit cornea. *J. Natl. Cancer Inst.* 52, 413-427.
- 33, 275 Gitay-Goren H., Soker S., Vlodavsky I. and Neufeld G. (1992). The binding of vascular endothelial growth factor to its receptors is dependent on cell surface associated heparin-like molecules. *J. Biol. Chem.* 267, 6093-6098.
- ng-term Goodman A.L. and Rone J.D. (1985) Detection of angiogenic (chemoattractant) activity released by rabbit luteal cells cultured in serum-free or serum-enriched media. *Biol. Reprod.* 32 (Suppl. 1), 296 (Abstract).
- JSA 76 Gospodarowicz D. (1983). Growth factors and their action in vivo and in vitro. *J. Pathol.* 141, 201-233.
- D. and Gospodarowicz D. and Cheng J. (1986). Heparin protects basic and acidic FGF from inactivation. *J. Cell. Physiol.* 128, 475-484.
- n-basic Gospodarowicz D., Cheng J., Liu G.M., Fujii D.K., Baird A. and Bohlen P. (1985) Fibroblast growth factor in human placenta. *Biochem. Biophys. Res. Commun.* 128, 554-562.
- . Am. J. Gospodarowicz D., Ferrara N., Schweigerer L. and Neufeld G. (1987). Structural characterization and biological functions of fibroblast growth factor. *Endocr. Rev.* 8, 95-114.
- action of Gospodarowicz D., Abraham J.A. and Schilling J. (1989). Isolation and characterization of vascular endothelial mitogen produced by pituitary-derived folliculo stellate cells. *Proc. Natl. Acad. Sci. USA* 86, 7311-7315.
- oplasia Goto F., Goto K., Weindel K. and Folkman J. (1993). Synergistic effects of vascular endothelial growth factor and basic growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gel. *Lab. Invest.* 69, 491-493.
- 1989b) Greenberg G. and Hunt T.K. (1978). The proliferative response in vitro of vascular endothelial and smooth muscle cells exposed to wound fluids and macrophages. *J. Cell. Physiol.* 97, 353-360.
- Science Greenberg G., Vlodavsky I., Foldart J.M. and Gospodarowicz D. (1980). Conditioned medium from endothelial cell cultures can restore the normal phenotypic expression of vascular endothelium maintained in vitro in the absence of fibroblast growth factor. *J. Cell. Physiol.* 103, 333-341.
- s. Proc. Greene H.S.N. (1943). The heterologous transplantation of embryonic mammalian tissues. *Cancer Res.* 3, 809-822.
- ial cell Grillo H.C. (1963). Origin of fibroblasts in wound healing: an autoradiographic study of inhibition of cellular proliferation by local X-irradiation. *Ann. Surg.* 157, 453-467.
- sement
- asts of
- estrous

- Groopman J.E., Gottlieb M.S. and Goodman J. (1984). Recombinant alpha-2 interferon therapy for Kaposi's sarcoma associated with the acquired immunodeficiency syndrome. *Ann. Intern. Med.* 100, 671-676.
- Gross J.L., Moscatelli D. and Rifkin D.B. (1983). Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro. *Proc. Natl. Acad. Sci. USA* 80, 2623-2627.
- Groszek E. and Grundy S.M. (1980). The possible role of the arterial microcirculation in the pathogenesis of atherosclerosis. *J. Chronic Dis.* 33, 679-684.
- Grunt T.W., Lametschwandter A., Karrer K. and Staindl O. (1986). The angioarchitecture of the Lewis lung carcinoma in laboratory mice. *Scanning Electron. Microsc.* 11, 557-573.
- Guillino P.M. (1981). Angiogenesis factor(s). In: *Handbook of experimental pharmacology*. Baserga R. (ed). Springer-Verlag. New York. p 427.
- Haar J.L. and Ackerman G.A. (1971). A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat. Rec.* 170, 199-224.
- Hadfield G. (1951). Granulation tissue. *Ann. Roy. Coll. Surg. Eng.* 9, 397-407.
- Hallahan T.W., Shapiro R. and Vallee B.L. (1991). Dual site model for the organogenic activity of angiogenin. *Proc. Natl. Acad. Sci. USA* 15, 2222-2226.
- Harris M.K. and Eakin R.M. (1949) Survival of transplanted ovaries in rats. *J. Exp. Zool.* 112, 131-163.
- Haynes W.L., Proia A.D. and Klintworth G.K. (1989). Effect of inhibitors of arachidonic acid metabolism on corneal neovascularization in the rat. *Invest. Ophthalmol. Vis. Sci.* 30, 1588-1593.
- Heder G., Jakob W., Halle W., Mauersberger B., Kambach G., Jentzsch K.D. and Oehme P. (1979). Influence of porcine corpus luteum extracts on DNA synthesis and proliferation of cultivated fibroblasts and endothelial cells. *Exp. Pathol.* 17, 493-497.
- Heimark R.L. and Schwartz S.M. (1988). The role of cell-cell interaction in the regulation of endothelial cell growth. In: *The Molecular and cellular biology and wound repair*. Clark R.A.F. and Henson P.H. (eds). Plenum. New York. p 359.
- Heldin P., Pertoft H., Nordlinder H., Heldin C-H. and Laurent T.C. (1991). Differential expression of platelet-derived growth factor alpha and beta receptors on fat-storing cells and endothelial cells of rat liver. *Exp. Cell Res.* 193, 364-369.
- Henkind P. (1978). Ocular neovascularization. The Krill memorial lecture. *Am. J. Ophthalmol.* 85, 287-301.
- Herbert J.M., Laplace M.C. and Maffrand J.P. (1988). Effect of heparin on the angiogenic potency of basic and acidic fibroblast growth factors in the rabbit cornea assay. *Int. J. Tissue React.* X, 133-139.
- Hertly M., Clark W.H., Rodeck U., Macianti M.L., Jambrosic J. and Koprowski H. (1987). Biology of tumor progression in human melanocytes. *Lab. Invest.* 56, 461-474.
- Herman I.M. and D'Amore P.A. (1985). Microvascular pericytes contain muscle and non-muscle actins. *J. Cell Biol.* 101, 43-52.
- Heuser L.S. and Miller F.N. (1986). Differential macromolecular leakage from the vasculature of tumors. *Cancer* 57, 461-464.
- His W. (1868). *Untersuchungen über die erste Anlage des Wirbelthierleibes*. F.C.W. Vogel. Leipzig.
- Hiti-Harper J., Wohl H. and Harper E. (1978). Platelet factor 4: An inhibitor of collagenase. *Science* 199, 991-992.
- Houck K.A., Ferrara N., Winer J., Cachianes G., Li B. and Leung D.W. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* 5, 1806-1814.
- Hsieh D.S.T., Langer R. and Folkman J. (1981). Magnetic modulation of release of macromolecules from polymers. *Proc. Natl. Acad. Sci. USA* 78, 1863-1867.
- Ide A.G., Baker N.H. and Warren S.L. (1939). Vascularization of the Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. *Am. J. Roentgenol.* 42, 891-899.
- Ingber D.E. and Folkman J. (1988). Inhibition of angiogenesis through modulation of collagen metabolism. *Lab. Invest.* 59, 44-51.
- Ingber D.E. and Folkman J. (1989). Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: Role of extracellular matrix. *J. Cell Biol.* 109, 317-330.
- Ingber D.E., Madri J.A. and Folkman J. (1986). A possible mechanism for inhibition of angiogenesis by angiostatic steroids: Induction of capillary basement membrane dissolution. *Endocrinology* 119, 1768-1775.
- Ingber D.E., Madri J.A. and Folkman J. (1987). Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. *In Vitro Cell Dev. Biol.* 23, 387-394.
- Ingber D., Fujita T., Kishimoto S., Sudo K., Kanamaru T., Brem H. and Folkman J. (1990). Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* 348, 555-557.
- Inomata H., Smelser G.K. and Polack F.M. (1971). Corneal vascularization in experimental uveitis and graft rejection-an electron microscopic study. *Invest. Ophthalmol.* 10, 840-850.
- Iruela-Arispe M.L., Bornstein P. and Sage H. (1991). Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. *Proc. Natl. Acad. Sci. USA* 88, 5026-5030.
- Ishikawa F., Miyazono K., Hellman U., Drexler H., Wernstedt C., Hagiwara K., Usuki K., Takaku F., Risau W. and Heldin C-H. (1989). Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature* 338, 557-562.
- Jaffe E. and Mosher D.F. (1978). Synthesis of fibronectin by cultured human endothelial cells. *J. Exp. Med.* 147, 1779-1791.
- Jaffe E.A., Nachman R.L., Becker C.G. and Minick C.R. (1972). Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J. Clin. Invest.* 51, 46a.
- Jaffe E., Nachman R.L., Becker C.G. and Minick C.R. (1973). Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52, 2745-2756.
- Jain R.K. (1985). Determinants of tumor blood flow. A review. *Cancer Res.* 48, 2641-2658.
- Jakob W., Jentzsch K.D., Mauersberger B. and Oehme P. (1977). Demonstration of angiogenesis activity in the corpus luteum of cattle. *Exp. Pathol.* 13, 231-236.
- Jennings M.A. and Florey H.W. (1970). Healing. In: *General pathology*. 4th ed. Florey H.W. (ed). Lloyd-Luke (Medical Books) Ltd., W.B. Saunders Co. Philadelphia. pp 480-548.
- Jensen H.M., Chen I., DeVault M.R. and Lewis A.R. (1982). Angiogenesis induced by "normal" human breast tissue: a probable marker for precancer. *Science* 218, 293-295.
- Joyce N.C., De Camilli P. and Boyles J. (1984a). Pericytes, like vascular smooth muscle cells, are immunocytochemical evidence for the

- presence of two isomyosins in graded concentrations. *J. Cell Biol.* 100, 1387-1395.
- Joyce N.C., De Camilli P. and Boyles J. (1984b). Pericytes, like vascular smooth muscle cells, are immunocytochemically positive for cyclic GMP-dependent protein kinase. *Microvasc. Res.* 28, 206-219.
- Joyce N.C., Haire M.F. and Palade G.E. (1985). Contractile proteins in pericytes. II. Immunocytochemical evidence for the presence of two isomyosins in graded concentrations. *J. Cell Biol.* 100, 1387-1395.
- Kalebic T., Garbisa S., Glaser B. and Liotta L.A. (1983). Basement membrane collagen: degradation by migrating endothelial cells. *Science* 221, 281-283.
- Kaminska G.M. and Niederkorn J.Y. (1993). Spontaneous corneal neovascularization in nude mice. Local imbalance between angiogenic and anti-angiogenic factors. *Invest. Ophthalmol. Vis. Sci.* 34, 222-230.
- Kaminski M., Majewski S., Jablonska S., and Pawinska, M. (1984). Lowered angiogenic capability of peripheral blood lymphocytes in progressive systemic sclerosis (scleroderma). *J. Invest. Derm.* 82, 239-243.
- Kanwar Y.S. and Farquhar M.G. (1979). Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. USA* 76, 1303-1307.
- Kanzani H., Okamura H., Takemori K. and Mori T. (1985) Ultrastructural changes of corpus luteum capillaries through its life span in the rabbit. *Biol. Reprod.* 32 (Suppl. 1), 295 (Abstract).
- Kardami K. and Frandrich R. (1989). Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J. Cell Biol.* 109, 1865-1875.
- Keck P.J., Hauser S.D., Krivi G., Sanzo K., Warren T., Feder J. and Connolly D.T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246, 1309-1312.
- Kiefer M., Stephens J.C., Crawford K., Okino K. and Barr P.J. (1990). Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* 87, 6985-6989.
- Klagsbrun M. and D'Amore P.A. (1991). Regulators of angiogenesis. *Annu. Rev. Physiol.* 53, 217-239.
- Klagsbrun M. and Folkman J. (1991). Angiogenesis. Peptide growth factors and their receptors II. Sporn M.B. and Roberts A.B. (eds). Springer-Verlag. New York. pp. 549-586.
- Klagsbrun M. and Vlodavsky I. (1988). Biosynthesis and storage of basic fibroblast growth factor (bFGF) by endothelial cells: Implication for the mechanism of action of angiogenesis. In: Growth factors and other aspects of wound healing: Biological and clinical implications. Riss A.R. Liss (ed). New York. pp 55-61.
- Klintworth G.K. (1977). The contribution of morphology to our understanding of the pathogenesis of experimentally produced corneal vascularization. *Invest. Ophthalmol. Vis. Sci.* 16, 281-285.
- Klintworth G.K. (1991). Hypotheses about the pathogenesis of corneal vascularization. In: Corneal angiogenesis. A comprehensive critical reviews. Klintworth G.K. (ed.) Springer-Verlag. Berlin. pp 23-29.
- Kocher O. and Madri J.A. (1989). Modulation of actin mRNAs in cultured vascular cells by matrix components and TGF-beta 1. *In Vitro Cell Dev. Biol.* 25, 424-434.
- Koester K. (1876). Endarteritis and arteritis. *Klin. Wochenschr.* 13, 454-455.
- Kohsaka S., Shinozaki T., Nakano Y., Takei K., Toya S. and Tsukada Y. (1989). Expression of Ia antigen on vascular endothelial cells in mouse cerebral tissue grafted into the third ventricle of rat brain. *Brain Res.* 484, 340-347.
- Koos R.D. (1986) Stimulation of endothelial cell proliferation by granulosa cell-conditioned medium. *Endocrinology* 119, 481-489.
- Koos R. and LeMaire W. (1983). Evidence for an angiogenic factor from rat follicles. In: Factors regulating ovarian function. Greenwald G.S. and Terranova P.F. (eds). Raven Press. New York. pp 191-195.
- Kubota Y., Kleinman H.K. Martin G.R. and Lawley T.J. (1988). Role of basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* 107, 1589-1598.
- Kuettner K.E., Croxen R.L., Eisenstein R. and Sorgente N. (1974). Proteinase inhibitor activity in connective tissue. *Experientia* 30, 595-597.
- Kuwabara T. and Cogan D.G. (1963). Retinal vascular patterns: IV. Mural cells of the retinal capillaries. *Arch. Ophthalmol.* 69, 492-502.
- Kull F.C. Jr., Brent D.A., Parikh I. and Cuatrecasas P. (1987). Chemical identification of a tumor-derived angiogenic factor. *Science* 236, 843-845.
- Kumar S., West D., Shahabaddin S., Arnold F., Haboubi N., Reid H. and Carr H. (1983). Angiogenesis factors from human myocardial infarcts. *Lancet* 2, 364-368.
- Langer R. and Folkman J. (1976). Polymers for the sustained release of proteins and other macromolecules. *Nature* 263, 797-800.
- Langer R., Brem H., Faltermann K., Klein M. and Folkman J.H. (1976). Isolation of a cartilage factor that inhibits tumor neovascularization. *Science* 193, 70-71.
- Langer R.S., Conn H., Vacanti J., Haudenschild C. and Folkman J. (1980). Control of tumor growth in animals by infusion of an angiogenesis inhibitors. *Proc. Natl. Acad. Sci. USA* 77, 4331-4335.
- Latker C.H. and Kuwabara T. (1981). Regression of the tunica vasculosa lentis in the postnatal rat. *Invest. Ophthalmol. Vis. Sci.* 21, 689-699.
- Latker C.H., Feinberg R.N. and Beebe D.C. (1986). Localized vascular regression during limb morphogenesis in the chicken embryo: II. Morphological changes in the vasculature. *Anat. Rec.* 214, 410-417.
- LeCompte P.M. (1967). Reactions of the vasa vasorum in vascular disease. In: Cowdry's arteriosclerosis, a survey of the problem. 2nd ed. Blumenthal H.T. (ed) Thomas C.C. Springfield. Illinois. pp 212-224.
- Lee A. and Langer R. (1983). Shark cartilage contains inhibitors of tumor angiogenesis. *Science* 221, 1185-1187.
- Leibovich S.J., Polverini P.J., Shepard H.M., Wiseman D.M., Shively V. and Nuseir N. (1987). Macrophage-induced angiogenesis is mediated by tumor necrosis factor alpha. *Nature* 329, 630-632.
- Leung D.W., Cachianes G., Kuang W-J., Goeddel D.V. and Ferrara N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306-1309.
- Leavesley D.I., Schwartz M.A., Rosenfeld M. and Cheres D.A. (1993). Integrin beta1 and beta3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J. Cell Biol.* 121, 163-170.
- Levy A., Tamargo R., Brem H. and Nathans D. (1989). An endothelial cell growth factor from the mouse neuroblastoma cell line NB41. *Growth Factors* 2, 9-19.
- Lewis W.H. (1925). The transformation of mononuclear blood cells into macrophages, epithelioid cells, and giant cells. *Harvy Lect.* 21, 77-112.
- Lewis W.H. (1931). The outgrowth of endothelium and capillaries in tissue culture. *Bull. Johns Hopk. Hosp.* 48, 242-253.

- Liaw L. and Schwartz S.M. (1993). Microtubule disruption stimulates DNA synthesis in bovine endothelial cells and potentiates cellular response to basic fibroblast growth factor. *Am. J. Pathol.* 143, 937-948.
- Lijnen H.R. and Collen D. (1986). Stimulation by heparin with plasmin-mediated conversion of single-chain to two-chain urokinase-type plasminogen activator. *Thromb. Res.* 43, 687-690.
- Liotta L. and Kleinerman J. and Saidel G. (1974). Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res.* 34, 997-1004.
- Lutty G.A., Thompson D.C., Gallup J.Y., Mello R.J., Patz A. and Fenselau A. (1983). Vitreous: an inhibitor of retinal extract-induced neovascularization. *Invest. Ophthalmol. Vis. Sci.* 24, 52-56.
- Maciag T. (1984). Angiogenesis. *Prog. Haemostat. Thromb.* 7, 167-182.
- Maciag T., Kadish J., Wilkins L., Sternerman M.B. and Weinstein R. (1982). Organizational behavior of human umbilical vein endothelial cells. *J. Cell Biol.* 94, 511-520.
- Madri J.A. and Pratt B.M. (1986). Endothelial cell-matrix interactions: In vitro models of angiogenesis. *J. Histochem Cytochem* 34, 85-91.
- Madri J.A. and Pratt B.M. (1988). Angiogenesis. In: *The Molecular and Cellular Biology of Wound Repair*. Clark R.A.F. and Henson P.M. (eds.). Plenum, New York. p 337.
- Madri J.A. and Stenn K.S. (1982). Aortic endothelial cell migration. I. Matrix requirements and composition. *Am. J. Pathol.* 106, 180-186.
- Madri J.A. and Williams S.K. (1983). Capillary endothelial cell cultures: matrix requirements and composition. 97, 153-156.
- Madri J.A., Williams S.K., Wyatt T., and Mezzio C. (1983). Capillary endothelial cell cultures: Phenotypic modulation by matrix components. *J. Cell Biol.* 97, 153-165.
- Madri J.A., Pratt B. and Tucker A. (1988). Phenotypic modulation of endothelial cells by transforming growth factor beta depends upon the composition and organization of the extracellular matrix. *J. Cell Biol.* 106, 1375-1384.
- Maione T.E., Gray G.S., Petro J., Hunt A.J., Donner A.L., Bauer S.I., Carson H.F. and Sharpe R.J. (1990). Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science.* 247, 77-79.
- Majewski S., Tigalowa M., Jablonska S., Polakowski I. and Janczura E. (1987). Serum samples from patients with active psoriasis enhance lymphocyte-induced angiogenesis and modulate endothelial cell proliferation. *Arch. Dermatol.* 123, 221-225.
- Makris A., Ryan K.J., Yasumizu T., Hill C.L. and Zetter B.R. (1984). The non luteal porcine ovary as a source of angiogenic activity. *Endocrinology* 115, 1672-1677.
- Markee J.E. (1940) Menstruation in intraocular endometrial transplants in the rhesus monkey. *Cont. Embryol.* 77, 221-308.
- Masferrer J.L., Rimarachin J.A., Gerritsen M.E., Falck J.R., Yadagiri P., Dunn M.W. and Laniado S.M. (1991). 12(R)-Hydroxyeicosatrienoic acid, a potent chemotactic and angiogenic factor produced by the cornea. *Exp. Eye Res.* 52, 417-424.
- Matsushashi K. (1961). Electron microscopic observation of the corneal vascularization. (Report I). *J. Clin. Ophthalmol.* 14, 121-127.
- Matsushashi K. (1962). Experimental study on the corneal vascularization. II. Electron microscope observation on the corneal vascularization. *Acta Soc. Ophthalmol. Jpn.* 66, 939-952.
- Maurice D.M., Zauberman H. and Michaelson I.C. (1966). The stimulus to neovascularization in the cornea. *Exp. Eye Res.* 5, 168-184.
- Mc Cracken J.S., Burger P.C. and Klintworth G.K. (1979). Morphologic observations on experimental corneal vascularization in the rat. *Lab. Invest.* 41, 519-530.
- Mc Donald R.A. (1959). Origin of fibroblasts in experimental healing wounds: autoradiographic studies using tritiated thymidine. *Surgery.* 46, 376-382.
- McKinney R.V. and Panner B.J. (1972). Regenerating capillary basement membrane in skeletal muscle wounds. *Lab. Invest.* 26, 100-113.
- Meyer J. (1852). Über die Neubildung von Blutgefäßen in plastischen Exsudaten seröser Membranen und in Hautwunden. *Ann. Charité (Berlin).* 4, 41-140.
- Miyazono K., Okabe T., Urabe A., Takaku F. and Heldin C-H. (1987). Purification and properties of an endothelial cell mitogen from human platelets. *J. Biol. Chem.* 262, 4098-4103.
- Montesano R. and Orci L. (1985). Tumor-promoting phorbol esters induce angiogenesis "in vitro". *Cell* 42, 469-477.
- Montesano R., Orci L. and Vassalli P. (1983). In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.* 97, 1648-1652.
- Montesano R., Vassalli J-D., Baird A., Guillemin R. and Orci L. (1986). Basic fibroblast growth factor induces angiogenesis in vitro. *Proc. Natl. Acad. Sci. USA* 83, 7297-7301.
- Moscatelli D. and Rifkin D.B. (1988). Membrane and matrix localization of proteases: A common theme in tumor invasion and angiogenesis. *Biochim. Biophys. Acta* 948, 67-85.
- Moscatelli D., Gross J.L. and Rifkin D.B. (1981). Angiogenic factors stimulate plasminogen activator and collagenase production by capillary endothelial cells. *J. Cell Biol.* 91, 201a (Abstract).
- Moscatelli D., Presta M. and Rifkin D.B. (1986). Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc. Natl. Acad. Sci. USA* 83, 2091-2095.
- Moses M.A., Sudhalter J. and Langer R. (1990). Identification of an inhibitor of neovascularization from cartilage. *Science* 248, 1408-1410.
- Murata J., Saiki I., Makabe T., Tsuta Y., Tokura S. and Azuma I. (1991). Inhibition of tumor-induced angiogenesis by sulfated chitin derivatives. *Cancer Res.* 51, 22-26.
- Murray I.C. and Leblond Ch.P. (1988). Immunoelectron microscopy of endothelial cells in rat incisor suggest that most basement membrane components are produced by young cells, whereas heparan sulfate proteoglycan is produced by both young and old cells. *J. Histochem. Cytochem.* 36, 763-773.
- Murray J.B., Brown L., Langer R. and Klagsbrun M. (1983). A microsustained release system for epidermal growth factor. *In Vitro* 19, 743-748.
- Muthukkaruppan V. and Auerbach R. (1979). Angiogenesis in the mouse cornea. *Science* 205, 1416-1418.
- Muthukkaruppan V.R., Kubal L. and Auerbach R. (1982). Tumor-induced neovascularization in the mouse eye. *J. Natl. Cancer Inst.* 69, 699-708.
- Nagy J.A., Brown L.F., Senger D.R., Lanir N., Van de Water L., Dvorak A.M. and Dvorak H.F. (1988). Pathogenesis of tumor stroma generation; critical role for leaky blood vessels and fibrin deposition. *Biochim. Biophys. Acta.* 948, 305-326.
- Nakayasu K. (1988). Origin of pericytes in neovascularization of rat cornea. *Jpn. J. Ophthalmol.* 32, 105-112.
- Nakayasu K., Hayashi N., Okisaka S. and Saro N. (1992). Formation of

capil
kerat
Nehls V.
peric
154.
Nehls V.
capill
469-
Nguyen
inhibi
saccl
2615
Nicosia
matri
ring-
Nicosia F
free r
in vit
Nicosia F
In vit
studie
Norrby K
angio
Virchi
Norrby K
gene:
Res.:
Norrby K
angio
30, 2:
Obeso J
quan
mono
Oikawa T
Okad.
83, 6-
Okamura
Kuwa
depei
necro
Biol. (
Olivo M.,
Flami
necro
and t
memt
Orlidge A
cell gi
1455-
Orlidge
cultur
ARVC
Paku S. &
exper
Paku S. .
genes
Pardanau
(1987
mono

- capillary-like tubes by vascular endothelial cells cocultivated with keratocytes. *Invest. Ophthalmol. Vis. Sci.* 33, 3050-3057.
- Nehls V. and Drenckhahn D. (1991). Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J. Cell Biol.* 113, 147-154.
- Nehls V., Denzer K. and Drenckhahn D. (1992). Pericyte involvement in capillary sprouting during angiogenesis in situ. *Cell Tissue Res.* 270, 469-474.
- Nguyen M., Folkman J. Bischoff J. (1992). 1-Deoxymannojirimycin inhibits capillary tube formation in vitro. Analysis of N-linked oligosaccharides in bovine capillary endothelial cells. *J. Biol. Chem.* 267, 26157-26165.
- Nicosia R.F. and Madri J.A. (1987). The microvascular extracellular matrix. Developmental changes during angiogenesis in the aortic ring-plasma clot model. *Am. J. Pathol.* 128, 78-90.
- Nicosia R.F. and Ottinetti A. (1990). Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. *Lab. invest.* 63, 115-122.
- Nicosia R.F., Tchao R. and Leighton J. (1982). Histiotypic angiogenesis in vitro. Light microscopic, ultrastructural and autoradiographic studies. *In Vitro* 18, 538-549.
- Norby K., Jakobsson A. and Sorbo J. (1986). Mast-cell-mediated angiogenesis: a novel experimental model using the rat mesentery. *Virchows Arch. (B)* 52, 195-206.
- Norby K., Jakobsson A. and Sorbo J. (1990a). Quantitative angiogenesis in spreads of intact rat mesenteric windows. *Microvasc. Res.* 39, 341-348.
- Norby K., Jakobsson A. and Sorbo J. (1990b). On mast-cell mediated angiogenesis in the rat mesenteric window assay. *Agents Actions* 30, 231-233.
- Obeso J.L. and Auerbach R. (1984). A new microtechnique for quantitating cell movement in vitro using polystyrene bead monolayers. *J. Immunol. Meth.* 70, 141-152.
- Okawa T., Suganuma M., Ashino-Fuse H. and Shimamura M. (1992). Okadaic acid is a potent angiogenesis inducer. *Jpn. J. Cancer Res.* 83, 6-9.
- Okamura K., Sato Y., Matsuda T., Hamanaka R., Ono M., Kohno K. and Kuwano M. (1991). Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. *J. Biol. Chem.* 266, 19162-19165.
- Olivo M., Bhardwaj R., Schulze-Osthoff K., Sorg C., Jacob H.J. and Flamme I. (1992). A comparative study on the effects of tumor necrosis factor alpha (TNF-alpha), human angiogenic factor (h-AGF) and basic fibroblast growth factor (bFGF) on the chorioallantoic membrane of the chick embryo. *Anat. Rec.* 234, 105-115.
- Orlidge A. and D'Amore P.A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.* 105, 1455-1462.
- Orlidge A. and D'Amore P.A. (1988). Endothelial cell-pericyte cocultures produce activated TGF-beta inhibits endothelial growth. *ARVO Abstracts. Invest. Ophthalmol. Vis. Sci.* 29 (Suppl.), 109-119.
- Paku S. and Lapis K. (1993). Morphological aspects of angiogenesis in experimental liver metastases. *Am. J. Pathol.* 143, 926-936.
- Paku S. and Paweletz N. (1991). First steps of tumor-related angiogenesis. *Lab. Invest.* 65, 334-346.
- Pardanaud L.C., Altmann C., Kito P., Dieterlenlievre F. and Buck C.A. (1987). Vasculogenesis in the early quail as studied with a monoclonal antibody recognizing endothelial cells. *Development* 100, 339-349.
- Passaniti A.P., Taylor A.M., Pilli R., Guo Y., Long P.V., Haney J.A., Pauly R.R., Grant D.S. and Martin G.R. (1992). A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab. Invest.* 67, 519-528.
- Paterson J.C. (1936). Vascularization and hemorrhage of the intima of arteriosclerotic coronary arteries. *Arch. Pathol.* 22, 313-324.
- Paterson J.C. (1938). Capillary rupture with intimal hemorrhage as a causative factor in coronary thrombosis. *Arch. Pathol.* 25, 474-487.
- Paweletz N. and Knierim M. (1989). Tumor-related angiogenesis. *Crit. Rev. Oncol/Hematol.* 9, 197-242.
- Peacock E.E. (1984). Wound repair. W.B. Saunders Co., Philadelphia. p 127.
- Pepper M.S. and Montesano R. (1990). Proteolytic balance and capillary morphogenesis. *Cell Diff. Dev.* 32, 319-328.
- Pepper M.S., Vassalli J.D., Montesano R. and Orci L. (1987). Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol.* 105, 2535-2541.
- Pepper M.S., Spray D.C., Chanson M., Montesano R., Orci L. and Meda P. (1989). Junctional communication is induced in migrating capillary endothelial cells. *J. Cell Biol.* 109, 3027-3038.
- Pepper M.S., Belin D., Montesano R., Orci L. and Vassalli J.D. (1990). Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J. Cell Biol.* 111, 743-755.
- Phillips H.S., Hains J., Leung W. and Ferrara N. (1990). Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 131, 254-260.
- Phillips G.D., Whitehead R.A. and Knighton D.R. (1991). Initiation and pattern of angiogenesis in wound healing in the rat. *Am. J. Anat.* 192, 257-262.
- Plouët J., Schilling J., Gospodarowicz D. (1989). Isolation and characterization of a newly identified endothelial cell mitogen produced by AIT-20 cells. *EMBO J.* 8, 3801-3806.
- Plunkett M.L. and Hailey J.A. (1990). An in vivo quantitative angiogenesis model using tumor cells entrapped in alginate. *Lab. Invest.* 62, 510-517.
- Polverini P.J. and Leibovich S.J. (1984). Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor-associated macrophages. *Lab. Invest.* 51, 635-642.
- Polverini P.J., Cotran R.S., Gimbrone M.A. Jr. and Unanue E.R. (1977a). Activated macrophages induce vascular proliferation. *Nature* 269, 804-806.
- Polverini P.J., Cotran R.S. and Sholley M.M. (1977b). Endothelial proliferation in the delayed hypersensitivity reaction: an autoradiographic study. *J. Immunol.* 118, 529-537.
- Presta M., Moscatelli D., Joseph-Silverstein J. and Rifkin D.B. (1986). Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol. Cell Biol.* 6, 4060-4066.
- Proia A.D., Chandler D.B., Haynes W.L., Smith C.F., Suvarnamani C., Erkel F.H. and Klintworth G.K. (1988). Quantitation of corneal neovascularization using computerized image analysis. *Lab. Invest.* 58, 473-479.
- Quinkler W., Maasberg M., Bernotat-Danielowski S., Luthe N., Sharma H. and Scharper W. (1989). Isolation of heparin-binding growth factors from bovine, porcine and canine hearts. *Eur. J. Biochem.*

- 181, 67-73.
- Raju K.S., Alessandri G., Ziohe M. and Gullino P.M. (1982). Ceruloplasmin copper ions and angiogenesis. *J. Natl. Cancer Inst.* 69, 1183-1188.
- Raju K.S., Alessandri G. and Gullino P.M. (1984). Characterization of a chemoattractant for endothelium induced by angiogenesis effectors. *Cancer Res.* 44, 1579-1584.
- Rastinejad F., Polverini P.J. and Bouck N.P. (1989). Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell* 56, 345-355.
- Ravindranath N., Little-Ihrig L., Phillips H.S., Ferrara N. and Zeleznick A.J. (1992). Vascular endothelial growth factor mRNA expression in the primate ovary. *Endocrinology* 131, 254-260.
- Real F.X., Oettgen H.F. and Krown S.E. (1986). Kaposi's sarcoma and the acquired immunodeficiency syndrome: treatment with high and low doses of recombinant leukocyte A interferon. *J. Clin. Oncol.* 4, 544-551.
- Reinhold H.S. and van den Berg-Blok A. (1984). Factors influencing the neovascularization of experimental tumours. *Biorheology* 21, 493-501.
- Rhine W., Hsieh D.S.T. and Langer R. (1980). Polymers for sustained macromolecule release: procedures to fabricate reproducible delivery systems and control release kinetics. *J. Pharm. Sci.* 69, 265-270.
- Rhodin J.A.G. and Fujita H. (1989). Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. *J. Submicrosc. Cytol. Pathol.* 21, 1-34.
- Ribatti D., Roncali L., Nico B. and Bertossi M. (1987). Effects of exogenous heparin on the vasculogenesis of the chorioallantoic membrane. *Acta Anat.* 130, 257-263.
- Richards J.S. (1980). Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiol. Rev.* 60, 51-89.
- Rifkin D.B. and Moscatelli D. (1989). Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.* 109, 1-6.
- Rifkin D.B., Gross J.L., Moscatelli D. and Jaffe E. (1982). Proteases and angiogenesis: Production of plasminogen activation and collagenase by endothelial cells. In: *Pathobiology of the endothelial cell*. Nossel H.L. and Vogel H.J. (eds). Academic Press Inc. New York. pp 191-197.
- Rios A., Mansell P.W., Newell G.R., Reuben J.M., Hersh E.M. and Guterman J.U. (1985). Treatment of acquired immunodeficiency syndrome-related Kaposi's sarcoma with lymphoblastoid interferon. *J. Clin. Oncol.* 3, 506-512.
- Risau W. (1986). Developing brain produces an angiogenesis factor. *Proc. Natl. Acad. Sci. USA.* 83, 3855-3859.
- Risau W. and Eklom P. (1986). Production of a heparin-binding angiogenesis factor by the embryonic kidney. *J. Cell Biol.* 103, 110-1107.
- Risau W. and Lemmon V. (1988). Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Dev. Biol.* 125, 441-450.
- Risau W., Sariola H., Zerwes H.G., Sasse J., Eklom P., Kemler R. and Doetschman T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell derived embryoid bodies. *Development* 102, 471-478.
- Roberts A.B., Sporn M.B., Assoian R.K., Smith J.M., Roche N.J., Wakefield L.M., Heine U.I., Liotta J.A., Falanga V., Kehrl J.H. and Fauci A.S. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA* 83, 4167-4171.
- Robertson N.E., Discapiani C.M., Downs E.C., Hailey J.A., Sarre O., Runkle R.L. Jr, Popper T.L. and Plunkett M.L. (1991). A quantitative in vivo mouse model used to assay inhibitors of tumor-induced angiogenesis. *Cancer Res.* 51, 1339-1344.
- Rofstad E.K. (1984). Growth and vascular structure of human melanoma xenografts. *Cell Tissue Kinet.* 17, 91-101.
- Rone J.D. and Goodman A.L. (1985). Detection of angiogenic activity from intact rabbit follicles cultured in serum-free medium. *Biol. Reprod.* 32 (Suppl. 1), 294 (Abstract).
- Rosenbruch M. (1989). Granulation tissue in the chick embryo yolk sac blood vessel system. *J. Comp. Pathol.* 101, 363-373.
- Ross R., Everett N.B. and Tyler R. (1970). Wound healing and collagen formation. VI. The origin of wound fibroblast studied in parabiosis. *J. Cell Biol.* 44, 645-654.
- Roth D.M., Maruoka Y., Rogers J., White F.C., Longhurst J.C. and Bloor C.M. (1987). Development of coronary collateral circulation in the left circumflex ameroid occluded swine myocardium. *Am. J. Physiol.* 253, H1279-H1288.
- Roth D.M., White F.C., Nichols M.L., Dobbs S.L., Longhurst J.C. and Bloor C.M. (1990). Effect of long-term exercise on regional myocardial function and coronary collateral development after gradual coronary artery occlusion in pigs. *Circulation* 82, 1778-1789.
- Rupnick M.A., Stokes C.L., Williams S.K. and Lauffenburger D.A. (1988). Quantitative analysis of random motility of human microvessel endothelial cells using a liner under-agarose assay. *Lab. Invest.* 59, 363-372.
- Ryan U.S. (1988). *Endothelial cells*. CRC Press, Boca Raton, Los Angeles.
- Ryan G.B. and Spector W.G. (1970). Macrophages turnover in inflamed connective tissue. *Proc. R. Soc. Lond. (Biol.)* 174, 269-292.
- Sabin F.R. (1920). Studies on the origin of the blood vessels and of red blood corpuscles as seen in the living blastoderm of chick during the second day of incubation. *Contr. Embryol.* 9, 215-262.
- Sandison J.C. (1928). Observations on the growth of blood vessels as seen in the transparent chamber introduced in the rabbit's ear. *Am. J. Anat.* 41, 475-496.
- Sasaki H., Hoshi H., Hong Y.-M., Suzuki T., Kato T., Saito M., Youki H., Karube K., Konno S., Onoderag M., Saito T. and Aoyagi S. (1989). Purification of acidic fibroblast growth factor from bovine heart and its localization in the cardiac myocytes. *J. Biol. Chem.* 264, 17606-17611.
- Sasaki K., Kiuchi Y., Sato Y. and Yamamori S. (1991). Morphological analysis of neovascularization at early stages of rat splenic autografts in comparison with tumor angiogenesis. *Cell Tissue Res.* 265, 503-510.
- Sasayama S. and Fujita M. (1992). Recent insights into coronary collateral circulation. *Circulation* 85, 1197-1204.
- Sato E., Ishibashi J. and Koide S.S. (1982). Inducement of blood vessel formation by ovarian extracts from mice injected with gonadotrophins. *Experientia* 38, 1248-1249.
- Sato N., Sawasaki Y., Senoo A., Fuse Y., Hirano Y. and Goto T. (1987). Development of capillary networks from rat microvascular fragments in vitro: The role of myofibroblastic cells. *Microvasc. Res.* 33, 194-210.
- Sato N., Fukuda K., Nariuchi H. and Sagara N. (1987). Tumor necrosis factor inhibiting angiogenesis in vitro. *J. Natl. Cancer Inst.* 79, 1383-1391.
- Sato N. Fre
stir
Sato Y
gro
act
121
Sato Y
by
tra
cul
Schap
art
6, 1
Schling
Ru
me
turr
Schling
P.C
exp
lam
J. F
Schmi
Am
gro
Cor
Schoef
The
97-
Schor
141
Schor
con
aort
Schreit
gro
gro
Schwar
gro
and
Schwei
nec
end
Selye
affe
120
Senger
Dvo
fact
987.
Senger
con
hurr
Shepro
mic
Res
Shing
Klaç

- 83, 4167. Sato N., Beitz J.G., Kato J., Yamamoto M., Clark J.W., Calabresi P. and Frackelton A.R. Jr. (1993). Platelet-derived growth factor indirectly stimulates angiogenesis *in vitro*. *Am. J. Pathol.* 142, 1119-1130.
- Sarre O. and Sato Y. and Rifkin D.B. (1988). Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J. Cell Biol.* 107, 1199-1205.
- of human Sato Y. and Rifkin D.B. (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor- β -like molecule by plasmin during coculture. *J. Cell Biol.* 109, 309-315.
- olic activity Schaper W. and Vandesteene R. (1987). The rate of growth of interarterial anastomoses in chronic coronary artery occlusion. *Life Sci.* 6, 1673-1680.
- ium. Biol. Schlingemann R.O., Rietveld F.J.R., de Waal R.M.W., Ferrone S. and Ruiters D.J. (1990). Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *Am. J. Pathol.* 136, 1393-1405.
- o yolk sac Schlingemann R.O., Rietveld F.J.R., Kwaspen F., van de Kerkhof P.C.M., de Waal R.M.W. and Ruiters D.J. (1991). Differential expression of markers for endothelial cells, pericytes, and basal lamina in the microvasculature of tumors and granulation tissue. *Am. J. Pathol.* 138, 1335-1347.
- collagen Schmidt M., Sharma H., Schott R.J. and Scharper W. (1991). Amplification and sequencing of mRNA encoding acidic fibroblast growth factor (aFGF) from porcine heart. *Biochem. Biophys. Res. Commun.* 180, 853-859.
- abiosis. J. Schoeff G.I. (1963). Studies on inflammation. III. Growing capillaries: Their structure and permeability. *Virchows Arch. Pathol. Anat.* 337, 97-141.
- and Blood Schor A.M. and Schor S.L. (1983). Tumour angiogenesis. *Br. J. Pathol.* 141, 385-413.
- in the left Schor A.M., Schor S.L. and Allen T.D. (1983). Effects of culture conditions on the proliferation morphology and migration of bovine aortic endothelial cells. *J. Cell Sci.* 62, 267-285.
- . Physiol. Schreiber A.B., Winkler M.E. and Derynck R. (1986). Transforming growth factor- α : a more potent angiogenic factor than epidermal growth factor. *Science* 232, 1250-1253.
- J.C. and Schwartz S.M., Gajdusek C.M. and Owens G.K. (1982). Vessel wall and anal myo growth control. In: *Pathobiology of the endothelial cells*. Nossel H.L. and Vogel H.J. (eds). Academic Press Inc. New York. pp 63-78.
- or gradual Schweigerer L., Malerstein B. and Gospodarowicz D. (1987). Tumor growth factor inhibits the proliferation of cultured capillary vessels as ear. Am. endothelial cells. *Biophys. Res. Commun.* 143, 997-1004.
- nger D.A. Selye H. (1953). On the mechanism through which hydrocortisone affects the resistance of tissues to injury. *J. Am. Med. Ass.* 152, 1207-1213.
- if human Senger D., Galli S.J., Dvorak A.M., Peruzzi C.A., Harvey V.S. and Dvorak H.F. (1983). Tumor cells secrete a vascular permeability assay aton. Los factor which promotes ascites fluid accumulation. *Science* 219, 983-987.
- in inflamed Senger D., Peruzzi C.A., Feder J. and Dvorak H.F. (1986). A highly and of red conserved vascular permeability factor secreted by a variety of during the human and rodent cell lines. *Cancer Res.* 46, 5629-5632.
- vessels as Schepers D. (1988). Endothelial cells, inflammatory edema, and the ear. Am. microvascular barrier: Comments by a "free radical". *Microvasc. Res.* 35, 247-264.
- Youki H. Shing Y., Folkman J., Haudenschild C., Lund D., Crum R. and S. (1989). Angiogenesis is stimulated by a tumor-derived 4, 17606 endothelial cell growth factor. *J. Cell Biochem.* 29, 275-287.
- hological t splenic Sholley M.M., Gimbrone M.A. and Cotran R.S. (1977a). Cellular issue Res. migration and replication in endothelial regeneration: a study using irradiated endothelial cultures. *Lab. Invest.* 36, 18-25.
- coronary Sholley M.M., Cavallo T. and Cotran R.S. (1977b). Endothelial cell od vessel proliferation in inflammation. I. Autoradiographic studies following ed with thermal injured to the skin of normal rats. *Am. J. Pathol.* 89, 277-296.
- Goto T. Sholley M.M., Gimbrone M.A. and Cotran R.S. (1978). The effects of ovascular asc. Res. leukocyte depletion on corneal neovascularization. *Lab. Invest.* 38, 32-40.
- ecrosis Res. Sholley M.M., Ferguson G.P., Seibel H.R., Montuor J.L. and Wilson J.D. 79, 1383. (1984). Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells. *Lab. Invest.* 51, 624-634.
- endothelial cell growth factor: *J. Cell Biochem.* 29, 275-287.
- Sholley M.M., Gimbrone M.A. and Cotran R.S. (1977a). Cellular migration and replication in endothelial regeneration: a study using irradiated endothelial cultures. *Lab. Invest.* 36, 18-25.
- Sholley M.M., Cavallo T. and Cotran R.S. (1977b). Endothelial cell proliferation in inflammation. I. Autoradiographic studies following thermal injured to the skin of normal rats. *Am. J. Pathol.* 89, 277-296.
- Sholley M.M., Gimbrone M.A. and Cotran R.S. (1978). The effects of leukocyte depletion on corneal neovascularization. *Lab. Invest.* 38, 32-40.
- Sholley M.M., Ferguson G.P., Seibel H.R., Montuor J.L. and Wilson J.D. (1984). Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells. *Lab. Invest.* 51, 624-634.
- Shweiki D., Itin A., Neufeld G., Gitay-Goren H. and Keshet E. (1993). Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J. Clin. Invest.* 91, 2235-2243.
- Sidky Y.A. and Borden E.C. (1987). Inhibition of angiogenesis by interferons: Effects on tumor- and lymphocyte-induced vascular responses. *Cancer Res.* 47, 5155-5161.
- Sillman F., Boyce J. and Fruchter R. (1981). The significance of atypical vessels and neovascularization in cervical neoplasia. *Am. J. Obstet. Gynecol.* 139, 154-159.
- Silverstein R.L., Leung L.K. and Nachman R.L. (1986). Thrombospondin: a versatile multifunctional glycoprotein. *Arteriosclerosis* 6, 245-253.
- Simionescu N. and Simionescu M. (1988). Endothelial cell biology in health and disease. Plenum Press. New York.
- Simionescu N. and Simionescu M. (1991). Endothelial cell dysfunction. Plenum Press. New York.
- Sims D.E. (1986). The pericyte. A review. *Tissue Cell* 18, 153-174.
- Skalli O., Pelte M-F., Peclet M-C., Gabbiani G., Gugliotta P., Bussolati G., Ravazzola M. and Orci L. (1989). Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37, 315-321.
- Smelser G.K. and Ozanics V. (1972). Modern views of corneal anatomy. In: *Symposium of the cornea*. Castroviejo R. (ed). C.V. Mosby Co. St. Louis. pp 127-143.
- Sorgente N., Kuettner K.E., Soble L.W. and Eisenstein R. (1975). The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibit invasion by vascularized mesenchyme. *Lab. Invest.* 32, 217-222.
- Soubrane G., Jordan J., Karpouzas I., Fayein N.A., Glaser B., Coscas G., Courtois Y. and Jeanny J.C. (1990). Binding of basic fibroblast growth factor to normal and neovascularized rabbit cornea. *Invest. Ophthalmol. Vis. Sci.* 31, 323-333.
- Spaniel-Borowski K. and Mayerhofer A. (1987). Formation and regression of capillary sprouts in corpora lutea of immature superstimulated golden hamsters. *Acta Anat.* 128, 227-235.
- Sparagen S.C., Bond V.P. and Dahl L.K. (1962). Role of hyperplasia in vascular lesions of cholesterol-fed rabbits studied with thymidine -H3 autoradiography. *Circ. Res.* 11, 329-336.
- Srivastava A., Laidler P., Hughes L.E., Woodcock J. and Shedden E.J. (1986). Neovascularization in human cutaneous melanoma: a quantitative morphological and Doppler ultrasound study. *Eur. J. Cancer Clin. Oncol.* 22, 1205-1209.

- Srivastava A., Laidler P., Davies R.P., Horgan K. and Hughes L.E. (1988). The prognostic significance of tumor vascularity. In intermediate-thickness (0.76-4.0 mm thick) skin melanoma: a quantitative histologic study. *Am. J. Pathol.* 133, 419-423.
- St. Clair D.K., Rybak S.M., Riordan J.F. and Vallee L. (1987). Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes. *Proc. Natl. Acad. Sci. USA* 84, 8330-8334.
- Starksen N.F., Harsh G.R. IV, Gibbs V.C. and Williams L.T. (1987). Regulated expression of the platelet-derived growth factor. A chain in microvascular endothelial cells. *J. Biol. Chem.* 262, 14381-14384.
- Stewart P.A. and Wiley M.J. (1981). Developing nervous tissues induced formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail-chick transplantation chimeras. *Dev. Biol.* 84, 183-192.
- Stokes C.L., Rupnick M.A., Williams S.K., and Laffenburger D.A. (1990). Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor. *Lab. Invest.* 63, 657-668.
- Sueishi K., Yasunaga C., Nakashima Y., Tsutsui H. and Ishii Y. (1989). Endothelium-fibrinolysis system interaction. *Nippon Ketsueki Gakkai Zasshi* 52, 1350-1358.
- Sugiura S. and Matsuda H. (1969). Electron microscopic studies on the corneal neo-vascularization. *Acta. Soc. Ophthalmol. Jpn.* 73, 1208-1221.
- Sunderkötter C., Goebeler M., Schulze-Osthoff K., Bhardwaj R. and Sorg C. (1991). Macrophage derived angiogenesis factors. *Pharmacol. Ther.* 51, 195-216.
- Szalay J. and Pappas G.D. (1970). Fine structure of rat corneal vessels in advanced stages of wound healing. *Invest. Ophthalmol.* 9, 354-365.
- Takigawa M., Enomoto M., Nishida Y., Pan H.-O., Kinoshita A. and Suzuki F. (1990a). Tumor angiogenesis and polyamines: α -difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits B16 melanoma-induced angiogenesis in ovo and the proliferation of vascular endothelial cells in vitro. *Cancer Res.* 50, 4134-4138.
- Takigawa M., Nishida Y., Suzuki F., Kishi J., Yamashita K. and Hayakawa T. (1990b). Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP and TIMP-2). *Biochem. Biophys. Res. Commun.* 171, 1264-1271.
- Tamargo R.J., Bok R.A. and Brem H. (1991). Angiogenesis inhibition by minocycline. *Cancer Res.* 51, 672-675.
- Tano Y., Chandler D.B. and Machener R. (1981). Vascular casts of experimental retinal neovascularization. *Am. J. Ophthalmol.* 92, 110-118.
- Tannock I.F. (1968). The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br. J. Cancer* 22, 258-276.
- Tannock I.F. (1970). Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. *Cancer Res.* 30, 2470-2476.
- Tannock I.F. and Hayashi S. (1972). The proliferation of capillary endothelial cells. *Cancer Res.* 32, 77-82.
- Taraboletti G., Roberts D., Lotta L.A. and Giavazzi R. (1990). Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: A potential angiogenesis regulatory factor. *J. Cell Biol.* 111, 765-772.
- Taylor S. and Folkman J. (1982). Protamine is an inhibitor of angiogenesis. *Nature* 297, 307-312.
- Terranova V.P., DiFlorio R., Lyall R.M., Hic S., Friesel R. and Maciag T. (1985). Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. *J. Cell Biol.* 101, 2330-2334.
- Thomas K., Rios Candelore M., Gimenez-Gallego G., Di Salvo J., Bennet C., Rodkey J. and Fluzpatrick S. (1985). Pure brain-derived acidic fibroblast growth factor is a potent vascular endothelial cell mitogen with sequence homology to interleukin 1. *Proc. Natl. Acad. Sci. USA* 82, 6409-6413.
- Thompson N., Bazoberry F., Speir E., Casscells W., Ferrans V., Flanders K., Kondaiah P., Geiser A. and Sporn M.B. (1988). Transforming growth factor beta-1 in acute myocardial infarction in rats. *Growth Factors* 1, 91-99.
- Thompson N.L., Flanders K.C., Smith J.M., Ellingsworth L.R., Roberts A.B. and Sporn M.B. (1989). Expression of transforming growth factor-beta 1 in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.* 108, 661-669.
- Tisher E., Mitchell R., Hartmann T., Silva M., Gospodarowicz D., Fiddes J. and Abraham J. (1991). The human gene for vascular endothelial growth factor. *J. Biol. Chem.* 266, 11947-11954.
- Tomaneck R.J., Schalk K.A., Marcus M.L. and Harrison D.G. (1989). Coronary angiogenesis during long-term hypertension and left ventricular hypertrophy in dogs. *Circ. Res.* 65, 352-359.
- Tseng S.C.G., Savion N., Gospodarowicz D. and Stern R. (1983). Modulation of collagen synthesis by a growth factor and by the extracellular matrix: Comparison of cellular response to two different stimuli. *J. Cell Biol.* 97, 803-809.
- Tseung S., Savion N., Stern R. and Gospodarowicz D. (1982). Fibroblast growth factor modulates synthesis of collagen in cultured vascular endothelial cells. *Eur. J. Biochem.* 122, 355-362.
- Tsuboi R., Sato Y. and Rifkin D.B. (1990). Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. *J. Cell Biol.* 110, 511-517.
- Tsuruoka N., Sugiyama M., Tawaragi Y., Tsujimoto M., Nishihara T., Goto T. and Sato N. (1988). Inhibition of in vitro angiogenesis by lymphotoxin and interferon-gamma. *Biochem. Biophys. Res. Commun.* 155, 429-435.
- Tuan D., Smith S., Folkman, J. and Merler E. (1973). Isolation of the nonhistone proteins of rat Walker carcinoma 256: their association with tumor angiogenesis. *Biochemistry* 12, 3159-3165.
- Unger E.F., Sheffield C.D. and Epstein S.E. (1991). Heparin promotes the formation of extracardiac to coronary anastomoses in a canine model. *Am. J. Physiol.* 260, H1625-H1634.
- Vaisman N., Gospodarowicz D. and Neufeld G. (1990). Characterization of the receptors for vascular endothelial growth factor. *J. Biol. Chem.* 265, 19461-19466.
- Verhoeven D. and Buyskens N. (1988). Desmin-positive stellate cells associated with angiogenesis in a tumour and non-tumour system. *Virchows Arch. (B)* 54, 263-272.
- Vlodavsky I. and Gospodarowicz D. (1979). Structural and functional alterations in the surface of vascular endothelial cells associated with the formation of a confluent cell monolayer and with the withdrawal of fibroblast growth factor. *J. Supramol. Struct.* 12 (Suppl. 1) 73-114.
- Vlodavsky I., Johnson L.K., Greenburg G. and Gospodarowicz D. (1979). Vascular endothelial cells maintained in the absence of fibroblast growth factor undergo structural and functional alterations that are incompatible with their in vivo differentiated properties. *J.*

C
Vlod
S
fi
e
2
Vrac
n
4
Wag
N
Wall
n
3
Wakt
o
A
Warr
bl
4
Warn
n
P
Warr
n
4
Warr
ai
E
Watt
ci
In
Webe
S
st
fr
vi
Weldi
ai
ca
West
in
1
White
of
th
White
(1
in
White
cc

- Cell Biol. 83, 468-486.
- Vlodavsky I., Folkman J., Sullivan R., Fridman R., Ishai-Michaeli R., Sasse J. and Klagsbrun M. (1987). Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into sub-endothelial extracellular matrix. *Proc. Natl. Acad. Sci. USA*, 84, 2292-2296.
- Vracko R. and Benditt E.P. (1970). Capillary basal lamina thickening: its relationship to endothelial cell death and replacement. *J. Cell Biol.* 47, 281-285.
- Wagner R.C. (1980). Endothelial cell embryology and growth. *Adv. Microcirc.* 9, 45-75.
- Wall R.T., Harker L.A. and Striker G.E. (1978). Human endothelial cell migration and stimulation by a released platelet factor. *Lab. Invest.* 39, 523-529.
- Wekul S. (1988). Two and three dimensional ultrastructural observation of two cell type angiogenesis in human granulation tissue. *Virchows Arch. (B)* 56, 127-139.
- Warren B.A. (1979a). The vascular morphology of tumors. In: *Tumor blood circulation*. Peterson H.I. (ed) CRC Press. Boca Raton. pp 1-47.
- Warren B.A. (1979b). Tumor angiogenesis. In: *Angiogenesis, vascular morphology and blood flow of experimental and human tumors*. Peterson H.I. (ed) CRC Press. Boca Raton. pp 33-74.
- Warren B.A. and Shubik P. (1966). The growth of blood supply to melanoma transplants in the hamster cheek pouch. *Lab. Invest.* 15, 464-478.
- Warren B.A., Greenblatt M. and Kommineni V.R.C. (1972). Tumor angiogenesis: ultrastructure of endothelial cells in mitosis. *Br. J. Exp. Pathol.* 53, 216-224.
- Watt S.L. and Auerbach R. (1986). A mitogenic factor for endothelial cells obtained from mouse secondary mixed leukocyte cultures. *J. Immunol.* 136, 197-202.
- Weber J., Meyer K.C., Banda P., Calhoun W.J. and Auerbach R. (1989). Studies of bronchoalveolar lavage cells and fluids in pulmonary sarcoidosis. II. Enhanced capacity of bronchoalveolar lavage fluids from patients with pulmonary sarcoidosis to induce cell movement in vitro. *Am. Res. Resp. Dis.* 140, 1450-1454.
- Weidner N., Semple J.P., Welch W.R. and Folkman J. (1991). Tumor angiogenesis and metastasis - Correlation in invasive breast carcinoma. *New Engl. J. Med.* 324, 1-8.
- West D.C., Hampson I.N., Arnold F. and Kumar S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science* 228, 1324-1326.
- White F.C., Sanders T.M. and Bloor C.M. (1978). Regional redistribution of myocardial blood flow after coronary occlusion and reperfusion in the conscious dog. *Am. J. Cardiol.* 43, 234-243.
- White C.W., Sondheimer H.M., Crouch E.C., Wilson H. and Fan L.L. (1989). Treatment of pulmonary hemangiomas with recombinant interferon alfa-2a. *New Engl. J. Med.* 320, 1197-1200.
- White F.C., Carroll S.M., Magnet A. and Bloor C.M. (1992). Coronary collateral development in the swine after coronary artery occlusion. *Circ. Res.* 17, 1490-1500.
- Williams R.J., Robertson D. and Davies A.J. (1989). Identification of vascular endothelial cells in murine omentum using the lectin, Dolichos biflorus agglutinin: possible applications in the study of angiogenesis. *Histochem. J.* 21, 271-278.
- Williams S. (1993). Angiogenesis in three-dimensional cultures. *Lab. Invest.* 69, 491-493.
- Winkles J.A., Friesel R., Burgess W.H., Howk R., Mehlman T., Weinstein R. and Moelag T. (1987). Human vascular smooth muscle cells both express and respond to heparin-binding growth factor 1 (endothelial cell growth factor). *Proc. Natl. Acad. Sci. USA* 84, 7124-7128.
- Yamagami I. (1970). Electronmicroscopic study on the cornea. I. The mechanism of experimental new vessel formation. *Jpn. J. Ophthalmol.* 14, 41-58.
- Yamaura H. and Sato H. (1973). Experimental studies on angiogenesis in AH109A ascites tumor tissue transplanted to a transparent chamber in rats. In: *Chemotherapy of cancer dissemination and metastasis*. Garattini S. and Franchi G. (eds) Raven. New York. pp 149-175.
- Yayon A., Klagsbrun M., Esko J.D., Leder P. and Ornitz D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64, 841-848.
- Young W.C. and Herman I.M. (1985). Extracellular matrix modulation of endothelial cell shape and motility following injury in vitro. *J. Cell Sci.* 73, 19-32.
- Zajchowski D.A., Band V., Trask D.K., Kling D., Connolly J.L. and Sager R. (1990). Suppression of tumor forming ability and related traits in MCF-6 human breast cancer cells by fusion with immortal mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* 2314-2318.
- Zauberman H., Michaelson I.C., Bergmann F. and Maurice D.M. (1969). Stimulation of neovascularization of the cornea by biogenic amines. *Exp. Eye Res.* 8, 77-83.
- Zetter B.R. (1980). Migration of capillary endothelial cells is stimulated by tumor derived factors. *Nature* 285, 41-43.
- Zetter B.R. (1988). Angiogenesis. State of the art. *Chest* 93, 159S-166S.
- Zhang Y., Cliff W.J., Schoeff G.I. and Higgins G. (1993). Immunohistochemical study of intimal microvessels in coronary atherosclerosis. *Am. J. Pathol.* 143, 164-172.
- Ziche M., Jones J. and Gullino P.M. (1982). Role of prostaglandin E1 and copper in angiogenesis. *J. Natl. Cancer Inst.* 69, 475-482.
- Ziche M., Ruggiero M., Pasquali F. and Chiarugi V.P. (1985). Effects of cortisone with and without heparin on angiogenesis induced by prostaglandin E1 and by S180 cells, and on growth of murine transplantable tumours. *Int. J. Cancer* 35, 549-552.
- Ziche M., Alessandri G. and Gullino P.M. (1989). Gangliosides promote the angiogenic response. *Lab. Invest.* 61, 629-634.
- Ziche M., Morbidelli L., Alessandri G. and Gullino P.M. (1992). Angiogenesis can be stimulated or repressed in vivo by a change in GM3:GD3 ganglioside ratio. *Lab. Invest.* 67, 711-715.

Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity

TAMAR ALON¹, ITZHAK HEMO², AHUVA ITIN¹, JACOB PE'ER², JONATHAN STONE³ & ELI KESHET¹

¹*Departments of Molecular Biology and ²Ophthalmology, Hebrew University–Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel*

³*Department of Anatomy and Histology, Sydney University, New South Wales 2006, Australia*
Correspondence should be addressed to E.K.

Retinopathy of prematurity (ROP) is initiated by hyperoxia-induced obliteration of newly formed blood vessels in the retina of the premature newborn. We propose that vessel regression is a consequence of hyperoxia-induced withdrawal of a critical vascular survival factor. We show that regression of retinal capillaries in neonatal rats exposed to high oxygen, is preceded by a shut-off of vascular endothelial growth factor (VEGF) production by nearby neuroglial cells. Vessel regression occurs via selective apoptosis of endothelial cells. Intraocular injection of VEGF at the onset of experimental hyperoxia prevents apoptotic death of endothelial cells and rescues the retinal vasculature. These findings provide evidence for a specific angiogenic factor acting as a vascular survival factor *in vivo*. The system also provides a paradigm for vascular remodelling as an adaptive response to an increase in oxygen tension and suggests a novel approach to prevention of ROP.

Retinopathy of prematurity (ROP) is a neovascularizing disease that causes blindness and that affects premature infants treated with high concentrations of oxygen. Despite careful monitoring of oxygen administered, the increased survival of ever more premature babies has produced a dramatic increase in the incidence of ROP in recent years¹. ROP development can be divided into two distinct stages: First, hyperoxia induces irreversible damage to immature retinal vessels of the neonate, resulting in intense retinal ischaemia. When the neonate resumes breathing normal air, the second phase is initiated, distinguished by excessive revascularization with abnormally leaky vessels, which frequently also leads to retinal detachment and loss of vision. The critical, initiating event in ROP pathogenesis is thus the physical obliteration of newly formed capillaries in the first stage.

Although it is generally believed that abnormal vasoproliferation in ROP (as well as in other forms of retinopathy) is caused by excessive production of an angiogenic factor elaborated by the ischaemic retina^{2,3} (presumably VEGF, refs 4–6), there is no mechanistic explanation as to why newly formed blood vessels of the retina are so vulnerable to an excess of oxygen.

We hereby propose that vessel regression in ROP represents an exaggeration of an otherwise natural response to oxygen surplus. During this adaptive process the tissue responds to excess oxygen by trimming the vascular tree so that the supply matches the metabolic requirements of the tissue. As a mechanism of hyperoxia-induced vessel regression, we suggest that maintenance of vascular networks, in general, depends on the continuous supply of a certain survival factor(s) and that hyperoxia is one possible way to downregulate vascular survival factor(s) to a level no longer sufficient for maintenance.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and an endothelial cell-specific mitogen^{7–10}. We and others

have previously identified VEGF as the linking factor between tissue hypoxia and a compensatory angiogenic response in the context of underperfused tumors^{11,12}. Subsequent studies have extended this observation to other ischaemic tissues, including the ischaemic retina^{6,13}. On the assumption that oxygen-regulated expression of VEGF is likely to be manifested also by a reduced level of expression in a tissue subjected to hyperoxia, VEGF seemed an excellent candidate for the role of survival factor proposed above.

Natural development of the retinal vasculature is considered to be regulated by oxygen and mediated by a vasoformative factor produced by the retina itself. We have recently shown that 'physiological' levels of hypoxia, caused by increasing demand for oxygen at the onset of neuronal activity, are detected by strategically located populations of neuroglia that secrete VEGF and induce the formation of retinal vessels. As the vessels become patent, the hypoxic stimulus is relieved, so vessel formation is matched to oxygen demand¹⁴. Other studies have shown that oxygen levels which, in the retina, can be manipulated by changing the oxygen content of inspired air, control the microarchitecture of retinal vessels, including capillary remodelling and formation of capillary-free zones¹⁵. Reasoning that the same oxygen-sensitive factor may promote both vascular expansion and regression, we examined whether hyperoxia-induced reduction in VEGF expression is responsible for vessel regression in a rat ROP model.

Hyperoxia induces regression of retinal capillaries by apoptosis
The timing and topography of vessel formation in the rat retina have been described^{14,16}. Unlike that in humans, where the vascular network of the retina develops during late fetal development, in rodents, the same process takes place mostly during the first two weeks of postnatal development. Briefly, the superficial layer of vessels forms first, extending across the surface of the retina, from the

optic disc towards the retina periphery. The process begins in late gestation and vessels spread steadily, reaching the edge of the retina by postnatal day 12 (P12). In a second process, vessels arise as buds from the superficial layer and grow radially outwards (towards the sclera), as far as the junction of the inner nuclear and outer plexiform layers. At this level, they extend branches parallel to the retinal surface, to form the deep layer of the retinal vasculature. To simulate the situation of a premature baby inspiring high oxygen in a rat model, we exposed newborn rats to an oxygen-enriched atmosphere during the second week of postnatal development. An elaborate network of blood vessels has already developed in the central part of the P7 retina (Fig. 1a). Exposure of P7 animals to 80% oxygen for 48 hours resulted in severe obliteration of the newly formed capillaries (Fig. 1b). The vascular network was visualized by incubating whole mounted retina preparations (wholemout retina) with an endothelial cell-specific lectin, followed by staining with an anti-lectin antibody conjugated to peroxidase. This procedure was preferred over angiographic techniques in order to distinguish effects of hyperoxia on vessel survival *per se* from possible effects on blood flow. For rats as for humans, vulnerability to high oxygen seems to depend on the degree of vessel maturation. Thus, in the P7 rat retina, hyperoxia led to regression of capillaries residing close to the optic disc but had only a minimal effect on more peripheral (less mature) capillaries (Fig. 1).

To determine whether capillary regression occurs via apoptosis — a programmed cell death due to withdrawal of survival factors — we examined the retinas of animals exposed to 80% oxygen for a relatively short period of 20 hours (that is, at a time closely preceding the physical elimination of capillaries) for the presence of cells undergoing apoptosis. To this end, the TUNEL procedure¹⁷ for *in situ* detection of nuclear DNA fragmentation (a hallmark of apoptosis) was adapted for analysis of wholemount retina preparations. Hyperoxia led to a massive apoptotic death of retinal cells (Fig. 1c; e, left; and f). Covisualization of the vascular network (Fig. 1d; e, right) and TUNEL-positive cells has identified the endothelial cells, mostly capillary endothelium, as the cells undergoing a selective hyperoxia-induced apoptosis. Notably, no TUNEL-positive cells were detected in major arteries and veins, consistent with the finding that these vessels survive the hyperoxic insult. A relatively high concentration of TUNEL-positive cells was detected in capillaries surrounding major arteries (Fig. 1e, f), consistent with the observation that hyperoxia led to widening of the capillary-free zones around major arteries (Fig. 1b).

Hyperoxia downregulates basal production of VEGF

We next examined our working hypothesis that hyperoxia-initiated vessel regression is due to downregulation of VEGF production to a level below that necessary to sustain newly formed capillaries. As a first step, we established that VEGF expression is indeed downregulated in cultured glial cells subjected to hyperoxia. Glia-derived cells were examined because they are the major source of retinal VEGF *in vivo* (see below). Using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, we showed that the low, constitutive level of VEGF messenger RNA that C6 glioma cells express when grown under normal (21%) oxygen (which is augmentable by hypoxia as previously shown and reconfirmed here) is reduced to a barely detectable level when the cells are grown under an oxygen-enriched atmosphere (Fig. 2, lanes 5–7). A similar regulation was observed by using primary cultures of astrocytes (Fig. 2, lanes 3 and 4).

To examine the effect of hyperoxia on retinal VEGF production *in vivo*, we compared the steady-state level of VEGF mRNA found in

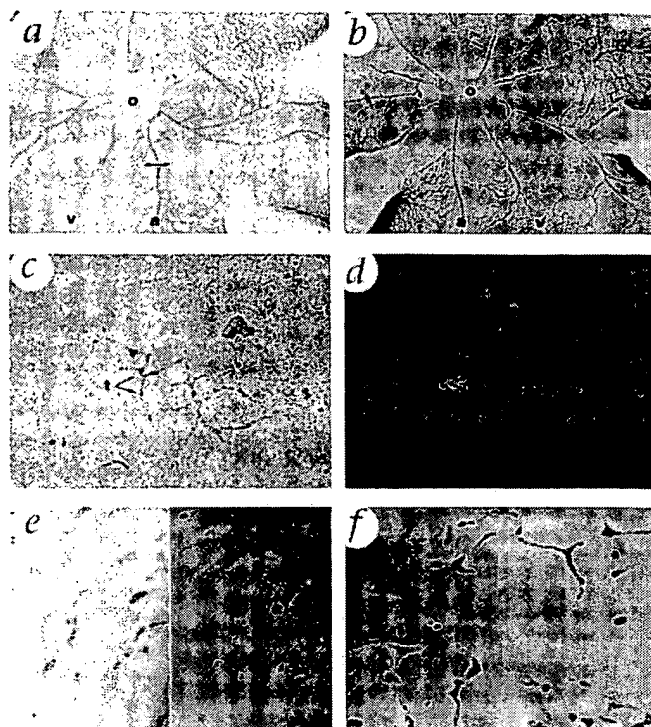


Fig. 1 Blood vessels and apoptotic cells visualized in whole mounted preparations of retinas exposed to hyperoxia. *a*, A control P7 retina. *b*, P9 retina from an animal exposed for two days (from P7 to P9) to 80% oxygen. The vascular tree was visualized by staining with an endothelial cell-specific lectin conjugated to peroxidase, as described in the Methods. Note the disappearance of capillaries that were present around the optic disc of a P7 retina. *c–f*, P9 retina that has been exposed for 20 hours to 80% oxygen. Apoptotic cells were visualized using an *in situ* TUNEL reaction (see Methods) (*c*, *e* left, *f*). Blood vessels in the same field of view were visualized using a lectin conjugated to an fluorescein isothiocyanate (FITC) fluorophore (*d*, *e* right). Arrows marked with a 't' point at representative TUNEL-positive cells, to illustrate that most of these cells are components of the vascular system. O, optic disc; a, artery; v, vein; z, capillary-free zone.

whole P9 control retinas with the level found in age-matched retinas of rats raised for the preceding two days in 80% oxygen. As shown in Fig. 2 (lanes 1 and 2), hyperoxia led to a significant downregulation of VEGF expression by the intact whole retina.

To identify VEGF-producing cells, a thin section of a P10 retina was hybridized *in situ* with a VEGF-specific probe. Three types of cells were identified as the major source of retinal VEGF: astrocytes distributed in the ganglion cell layer, Müller cells embedded in the inner nuclear layer, and retinal pigmented epithelium (RPE) residing at the outer boundary of neural retina (Fig. 3, *a*). The spatial distribution of these cells is consistent with the notion that the targets of the VEGF produced are nearby vessels composing the superficial network, the deeper plexus, and the choriocapillary system, respectively. The notion that VEGF produced by these cell acts on nearby vessels in a paracrine fashion is supported by earlier findings showing that retinal endothelial cells express the cognate VEGF receptor, flk-1 (ref. 14). Note that blood vessels have already been formed in the segment of the retina shown, suggesting that ongoing expression of VEGF may serve a function distinct from that of attracting new blood vessels to that area.

To identify which of the VEGF-producing cells downregulates VEGF expression in response to hyperoxia in the context of the intact retina *in vivo*, a retina of a P10 control animal and an age-matched retina from an animal raised for three days (P7 to P10) in 80% oxygen were hybridized *in situ* with a VEGF-specific probe. Whereas expression of VEGF mRNA by RPE cells was unaffected, expression of VEGF by the two types of neuroglial cells, namely astrocytes and Müller cells, was downregulated in response to hyperoxia (Fig. 3).

VEGF protects the retina from hyperoxia-induced regression

The experiments described above have clearly demonstrated that raising newborn rats in an oxygen-enriched atmosphere leads to downregulation of VEGF production by neuroglial cells, on the one hand, and to capillary regression on the other hand. To establish a cause-and-effect relationship between VEGF withdrawal and apoptotic death of endothelial cells, we examined whether exogenous VEGF can compensate for the deficit in endogenous VEGF and rescue the vasculature. One eye of each tested animal was injected intraocularly with purified recombinant VEGF₁₆₅ before the animal was placed in an 80% oxygen chamber. Animals were killed 20 hours or 48 hours later, and retinas processed to visualize cells undergoing apoptosis or to visualize the vascular tree, respectively. It should be pointed out that direct comparison of the retina resected from the VEGF-injected eye with the retina resected from the mock-injected eye of the same animal assured that the differences observed were not due to differences in developmental age, or to biological variability among littermates, or to variations in experimental conditions.

As shown in Fig. 1, in non-VEGF-treated eyes, hyperoxia led to massive endothelial cell apoptosis and disappearance of capillaries around the optic disc (see also Fig. 4a, c). Although TUNEL-positive cells are distributed throughout the retina and may include non-endothelial cells, massive cell death is mostly observed in the same area that eventually (one day later) will be 'cleared' from most capillaries. In contrast, eyes injected with VEGF showed a reduced endothelial cell apoptosis around the optic disc and maintained the normal vascular architecture. In particular, VEGF fully protected the capillaries around the optic disc from destruction (Fig. 4d).

Discussion

The results shown above demonstrate that under conditions leading to withdrawal of VEGF from a tissue that normally expresses it, selective apoptosis of endothelial cells occurs, which results in the regression of the newly formed capillary network. The working hypothesis that, in addition to its established role as an endothelial cell-specific mitogen, VEGF may act as a vascular survival factor, was proven by showing that exogenous VEGF compensated for endogenous VEGF withdrawal and rescued the vasculature from destruction.

An alternative interpretation of the results, namely, that VEGF did not prevent vessel regression but, instead, promoted revascularization after vessels have regressed, seems unlikely for the following reasons: First, exogenous VEGF reduced the degree of endothelial cell apoptosis in retinas exposed to high oxygen (compare Fig. 4a, b). Second, the microarchitecture of retinal vessels in the VEGF-injected eyes was indistinguishable from that of an age-matched retina that has never been exposed to hyperoxia, including the preservation of capillary-free zones around major arteries (compare Figs. 1a and 4d). This is in sharp contrast to the loss of normal vascular organization that characterizes ischaemia-induced (and

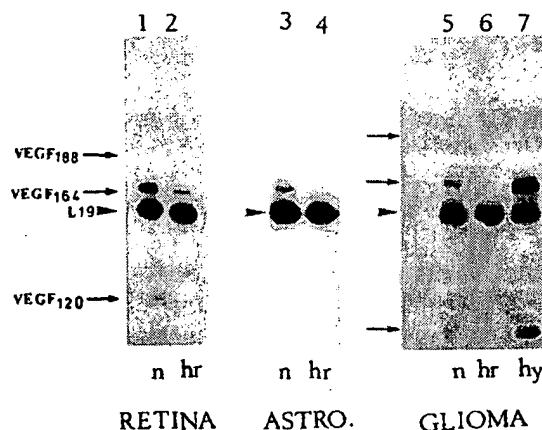


Fig. 2 A quantitative reverse transcriptase-PCR analysis of VEGF mRNAs in intact retina, primary astrocytes and glioma cultures. See Methods for the preparation of whole retinas, cell cultures, and for experimental procedures. Lanes 1 and 2 show VEGF cDNA fragments amplified from RNAs extracted from a pool of control P9 retinas or from a pool of P9 retinas that were exposed to 80% oxygen for 2 days, respectively. Amplified VEGF cDNA fragments (indicated by arrows) are 297 base pairs (bp), 225 bp, and 99 bp long (encoding VEGF₁₈₈, VEGF₁₆₄, and VEGF₁₂₀ respectively). Arrowhead points to the 194-bp-long fragment of coamplified ribosomal L19 cDNA. n, cells grown under normoxia; hr, cells grown under hyperoxia; hy, cells grown under hypoxia.

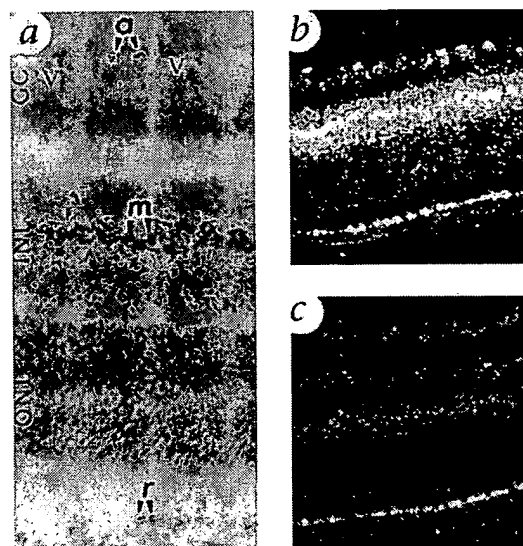


Fig. 3 Identification of VEGF-producing cells by *in situ* hybridization. a, A P10 retina section hybridized with a VEGF-specific probe. A strong hybridization signal is detected in astrocytes (a) located in the most superficial layer of the ganglion cell layer (GC), in the vicinity of blood vessels (V); VEGF mRNA is also detected in Müller cells (m) residing at the middle of the inner nuclear layer (INL), and in retinal pigmented epithelium cells (r) located at the junction of the retina and underlying choroid. b and c, Dark-field images of a P10 retina section (b) and a section of a P10 retina that has been exposed to hyperoxia for three days (c). Note that, in comparison with the grossly similar hybridization signal detected in the retinal pigmented epithelium (marked by an arrowhead), VEGF mRNA in astrocytes and Müller cells was downregulated in the hyperoxic retina.

presumably VEGF-mediated) neovascularization associated with human retinopathies and in animal models of ROP (refs 18, 19).

The notion that vascular networks may depend on the continuous presence of certain maintenance factors for their survival is supported by several lines of evidence. First, there is a growing awareness of the fact that growth factors, in general, may also function as survival factors²⁰ (such as, nerve growth factors). Second, certain vascular networks regress normally during development (for example, the hyaloid vascular system of the eye regresses about the same time that the retinal network develops), and it is likely that regression of transient vascular networks is caused by downregulation of essential survival factor(s). Third, there is evidence that withdrawal of fibroblast growth factor (FGF) from the medium of aorta explant cultures results in regression of preformed microvascular sprouts²¹. This study, however, provides the first *in vivo* evidence for an angiogenic growth factor functioning as an endothelial cell survival factor.

Regulation of angiogenic factor production by oxygen tension is a unique feature of VEGF, distinguishing it from most other known angiogenic factors. Previous studies have linked hypoxia-inducibility of VEGF to vascular expansion in poorly perfused tissues. Here we highlight the reverse situation, namely, how VEGF, repressed by hyperoxia, mediates a feedback response of vascular trimming in situations where fewer vessels may be sufficient to satisfy the metabolic demands of the tissue. Thus, three situations may exist: (1) under normoxia VEGF is produced at a level required to sustain existing vessels. (2) When the tissue is inadequately perfused, hypoxia leads to upregulation of VEGF to its 'angiogenic dose'. (3) In hyperoxia, VEGF expression is downregulated to a level below the 'maintenance dose' and, hence, some vessels may regress. An example for the last case may be found during normal development of the retinal network. This process, which we have recently shown to be mediated by VEGF (ref. 14), is characterized by exuberant development of blood vessels and their subsequent trimming after the initiation of blood flow.

An efficient mechanism for adjusting vascular density to situations of changing requirements is particularly important in neuronal tissues. This is because neuronal tissues consume more oxygen than other tissues and that the intensity of neuronal activity may frequently change. We believe that the results shown here for the neurosensory retina, including the specialized role of neuroglial cells in producing the angiogenic/survival factor, also apply to the brain, in general.

The initiating event in ROP pathogenesis is obliteration of newly formed capillaries in response to an extended exposure to high oxygen. Following Ashton's initial suggestion³ that many of the pathologic responses to the retinal vessels are not separate morbid processes, but exaggerations of normal behaviour, we suggest that capillary regression in ROP represents an exaggeration of the adaptive response of hyperoxia-induced vascular trimming, mediated by the withdrawal of the vascular survival factor VEGF. Capillary regression results in severe retinal non-perfusion and elicits ischaemia-induced neovascularization. As suspected, recent studies have shown that VEGF is upregulated in the ischaemic retina in different types of retinopathy⁴⁻⁶, including animal models of ROP (refs 22, 23), and it has been suggested that excessive production of VEGF is responsible for abnormal vasoproliferation associated with these diseases. Thus, VEGF may play two distinct roles in ROP pathogenesis: controlling vessel regression during the first phase and inducing neovascularization in the second phase. Since the recognition of VEGF as the long-sought vasoformative factor underlying retinopathies, it became clear that antagonizing

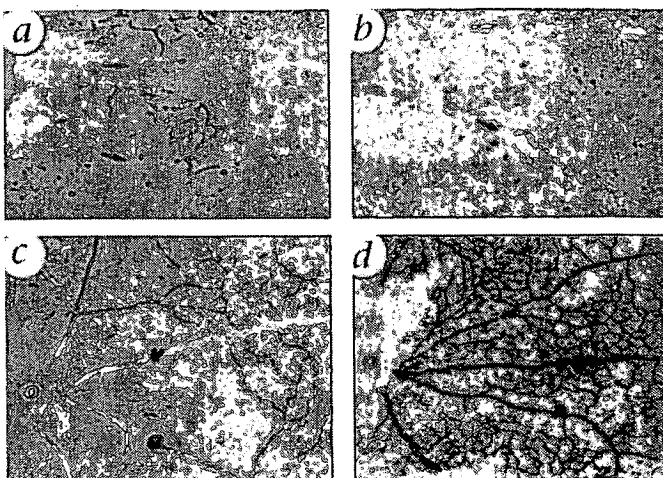


Fig. 4 Effect of exogenous VEGF on endothelial cell apoptosis and vessel survival. The left eye of each anaesthetized P7 animal was injected with 70 ng of recombinant VEGF₁₆₅ (produced by a baculovirus vector in an insect cell line, a generous gift from G. Neufeld) in 1 μ l sterile saline. The right eye was injected with saline alone. Animals were immediately placed, together with their mothers in a chamber under a constant flow of 80% oxygen. Then, 20 hours later (a and b) or 48 hours later (c and d) eyes were enucleated and wholemount retina preparations were analysed for TUNEL-positive cells or for the status of the vascular tree, respectively. Left and right figures show, in each case, the control and VEGF-injected eye, respectively, of the same animal. Results shown in c and d are representative of results obtained in all ten animals analysed. Arrows in the top portions of the figure indicate the locations of adjacent vein and artery branching from the optic disc, to illustrate apoptosis of interconnecting capillaries. o, optic disc; v, vein; a, artery.

VEGF action during the proliferative phase of the disease might be of a therapeutic value. We submit that a better way to prevent ROP would be to 'rescue' vessels from oxygen-induced damage in the first place. This may be achieved through injection of VEGF before subjecting the baby to high oxygen. Experiments presented here, using a rat ROP model, suggest that VEGF administered at this time may indeed protect retinal vessels from the hyperoxia-induced damage. Further experiments are, of course required to examine the potential therapeutic benefits of this approach.

Methods

Animals, wholemount retina preparations and cultured cells. Sabra albino rats aged P (postnatal day) 0 to 14 were used. Animals were killed using an overdose (60 μ g g⁻¹) of sodium pentobarbitone, and eyes were immediately enucleated.

For *in situ* hybridizations, whole globes were fixed in buffered formalin for 1–2 days. For preparation of wholemount retinas, enucleated globes were first fixed in 4% paraformaldehyde in PBS for 2 h. Retinas were then dissected in PBS by carefully cleaning the sclera and choroid from the outer surface of the retina and by removing the hyaloid vessels and vitreous humour from the inner surface.

Primary astrocytes were isolated from neonatal rat brain as previously described²⁴. Astrocytes and C6 cells, a clonal glial cell line derived from a rat glial tumor, were grown in Dulbecco-modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and antibiotics.

Exposure to hyperoxia. Some animals were raised in 80% oxygen for 1–3 days, with their mothers; the remainder were littermate controls

raised in normoxia. The oxygen-enriched atmosphere was obtained by mixing air and oxygen. Oxygen levels were monitored by a polarographic oxygen sensor (Commonwealth Industrial Gases, Australia).

Cells were subjected to hyperoxia by placing culture dishes in a 37 °C incubator equilibrated with a mixture of 95% oxygen/5% CO₂. To achieve hypoxia, near confluent cultures were incubated in a GasPak Plus anaerobic culture chamber (BBL Microbiology Systems), utilizing hydrogen and a palladium catalyst to remove all traces of oxygen. Cell cultures were exposed to either hyperoxia or hypoxia for 18 h.

Visualization of blood vessels by lectin immunohistochemistry. Retinas were prepared for immunohistochemistry as previously described¹⁴. Briefly, radial incisions were made to permit flattening of the retina, which was then fixed by immersion in 0.5% paraformaldehyde, post-fixed in cold 70% ethanol, and washed in PBS containing 1% Triton X-100. Blood vessels were visualized by incubation with the *Bandeira simplicifolia* isolectin B4 (biotin-conjugated, Sigma) for 2 h, followed by incubation with Extra-Avidin-peroxidase (BioMakor, Rehovot; 1:20 dilution) and staining with 3-amino-9-ethylcarbazole (AEC). In cases where blood vessels were covisualized with TUNEL-positive cells, retinas were reacted with a *Bandeira simplicifolia* isolectin B4 conjugated to FITC (Sigma), and vessels were visualized by fluorescence.

TUNEL (terminal deoxynucleotidyl transferase (TdT)-assisted, dUTP (digoxigenin nick end labelling)) analysis of wholemount retinas. TUNEL-positive cells in fixed and rehydrated wholemount retinas were identified *in situ* using the method of Gavrieli *et al.*¹⁷. Briefly, retinas were first treated with 3% H₂O₂ to inactivate endogenous peroxidase, and then incubated for 60 min at 37 °C with 0.3 e.u. µl⁻¹ terminal-deoxynucleotidyl-transferase (TdT) (Boehringer) in a solution containing 30 mM Tris base, 140 mM sodium cacodylate pH 7.2, 1 mM cobalt chloride and 40 µM biotinylated-11-dUTP. The reaction was terminated by transferring the retinas to 0.3 M NaCl/30 mM sodium citrate. Following a block with 2% bovine serum albumin and extensive washing in PBS, retinas were incubated with Extra-Avidin and stained with AEC, as described above.

In situ hybridization. Paraffin-embedded eyes were sectioned (5–10 µm thick), processed and hybridized *in situ* as previously described¹¹. Autoradiographic exposure was for 5–9 days. As a VEGF-specific probe, we used a 1.8-kb-long cDNA fragment containing approximately the 3' two-thirds of the coding region, as well as the 3'-untranslated region of mouse VEGF₁₆₅. cDNA was subcloned onto the polylinker of a PBS vector (Stratagene) and were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ³²S-labelled complementary RNA in either the antisense or sense orientation (using T3 or T7 RNA polymerase, respectively). The RNA probe was fragmented by mild alkaline treatment prior to use in *in situ* hybridization.

Reverse transcriptase-PCR analysis. Complementary DNA was synthesized from 300 ng of total RNA using oligo(dT) as a primer and AMV reverse transcriptase. PCR amplification was carried out in the presence of a [³²P]dCTP tracer (2 µCi in a 100 µl reaction volume), 1 mM of each dNTP, 2.5 mM MgCl₂, and 2.5 units of *Taq* polymerase. Twenty-five amplification cycles were used, each consisting of a 1-min incubation at 94 °C, a 2-min incubation at 65 °C, and a 3-min incubation at 72 °C. cDNAs were coamplified with a pair of VEGF-specific oligonucleotides and a pair of L19 ribosomal protein-specific oligonucleotides (serving as an internal standard). The following oligonucleotides were used: (1) GGAGAGAT-GAGCTTCTACAG and TCACCGCCTTGGCTTGTCACA, corresponding to amino acids 92–98 and to the six carboxy-terminal amino-acids of VEGF, respectively; (2) CTGAAGGTCAAAGGGAAT-

GTG and GGACAGACTCTTGATGATCTC, corresponding to forward and reverse sequences, respectively, of L19. Amplified fragments were resolved in 6% non-denaturing polyacrylamide gel and images were obtained by autoradiography.

Acknowledgements

This research was supported by the Israel Science Foundation (administered by the Israel Academy of Sciences and Humanities and Charles H. Revson Foundation) and by the March of Dimes Foundation.

RECEIVED 30 MARCH; ACCEPTED 11 AUGUST 1995

- Gibson, D.L., Sheps, S.B., Uh, S.H., Schechter, M.T. & McCormick, A.Q. Retinopathy of prematurity-induced blindness: Birth weight-specific survival and the new epidemic. *Pediatrics* 86, 405–412 (1990).
- Michaelson, I.C. The mode of development of the vascular system of the retina: With some observations on its significance for certain retinal diseases. *Trans. Ophthalmol. Soc. U.K.* 68, 137–80 (1948).
- Ashton, N., Ward, B. & Serpell, G. Effect of oxygen on developing retinal vessels with particular reference to the problem of retrolental fibroplasia. *Br. J. Ophthalmol.* 38, 397–431 (1954).
- Adamis, A.P. *et al.* Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am. J. Ophthalmol.* 118, 445–450 (1994).
- Aiello, L.P. *et al.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *New Engl. J. Med.* 331, 1480–1487 (1994).
- Pe'er, J. *et al.* Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab. Invest.* 72, 638–645 (1995).
- Gospodarowicz, D., Abraham, J.A. & Schilling, J. Isolation and characterization of vascular endothelial mitogen produced by pituitary-derived folliculo stellate cells. *Proc. natn. Acad. Sci. U.S.A.* 86, 7311–7315 (1989).
- Keck, P.J. *et al.* Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246, 1309–1312 (1989).
- Leung, D.W., Cachianes, G., Kaung, W.J., Goeddel, D.V. & Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306–1309 (1989).
- Dvorak, H.F. *et al.* Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: Concentration in tumor blood vessels. *J. exp. Med.* 174, 1275–1278 (1991).
- Shweiki, D., Itin, A., Soffer, D. & Keshet, E. Vascular endothelial growth factor induced by hypoxia mediates hypoxia-initiated angiogenesis. *Nature* 359, 843–845 (1992).
- Plate, K.H., Breier, G., Wiedh, H.A. & Risau, W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* 359, 845–848 (1992).
- Miller, J.W. *et al.* Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. *Am. J. Ophthalmol.* 145, 574–578 (1994).
- Stone, J. *et al.* Development of retinal vasculature is mediated by hypoxia-induced endothelial growth factor expression by neuroglia. *J. Neurosci.* 15, 4738–4747 (1995).
- Phelps, D.L. Oxygen and developmental retinal capillary remodeling in the kitten. *Invest. Ophthalmol. Vis. Sci.* 31, 2194–2200 (1990).
- Ashton, N. & Blach, R. Studies on developing retinal vessels. VIII. Effect of oxygen on the retinal vessels of the ratling. *Br. J. Ophthalmol.* 45, 321–340 (1961).
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S.A. Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501 (1992).
- Chan-Ling, T., Tout, S., Holländer, H. & Stone, J. Vascular changes and their mechanisms in the feline model of retinopathy of prematurity. *Invest. Ophthalmol. Vis. Sci.* 33, 2128–2147 (1992).
- Penn, J.S., Tolman, B.L. & Lowery, L.A. Variable oxygen exposure causes pre-retinal neovascularization in the newborn rat. *Invest. Ophthalmol. Vis. Sci.* 34, 576–585 (1993).
- Raff, C.M. Social controls on cell survival and cell death. *Nature* 356, 397–399 (1992).
- Villaschi, S. & Nicosia, R.F. Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury. *Am. J. Pathol.* 143, 181–190 (1993).
- Pierce, A.P., Avery, R.L., Foley, E.D., Aiello, L.P. & Smith, L.E.H. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc. natn. Acad. Sci. U.S.A.* 92, 905–909 (1995).
- Stone, J. *et al.* The role of vascular endothelial growth factor and astrocyte degeneration in the pathogenesis of retinopathy of prematurity. *Vis. Sci.* (in the press).
- Frangakis, M. V. & Kimelberg, H.M. Dissociation of neonatal rat brain by dispase for the preparation of primary astrocyte cultures. *Neurochem. Research* 9, 1689–1698 (1984).

Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: Induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal

(neovascularization/vessel regression/hemorrhage/tetracycline-inducible expression)

LAURA E. BENJAMIN AND ELI KESHET

Department of Molecular Biology, Hadassah Medical School, The Hebrew University, Jerusalem 91120, Israel

Communicated by M. Judah Folkman, Harvard Medical School, Boston, MA, May 6, 1997 (received for review February 20, 1997)

ABSTRACT We have recently shown that VEGF functions as a survival factor for newly formed vessels during developmental neovascularization, but is not required for maintenance of mature vessels. Reasoning that expanding tumors contain a significant fraction of newly formed and remodeling vessels, we examined whether abrupt withdrawal of VEGF will result in regression of preformed tumor vessels. Using a tetracycline-regulated VEGF expression system in xenografted C6 glioma cells, we showed that shutting off VEGF production leads to detachment of endothelial cells from the walls of preformed vessels and their subsequent death by apoptosis. Vascular collapse then leads to hemorrhages and extensive tumor necrosis. These results suggest that enforced withdrawal of vascular survival factors can be applied to target preformed tumor vasculature in established tumors. The system was also used to examine phenotypes resulting from over-expression of VEGF. When expression of the transfected VEGF cDNA was continuously "on," tumors became hyper-vascularized with abnormally large vessels, presumably arising from excessive fusions. Tumors were significantly less necrotic, suggesting that necrosis in these tumors is the result of insufficient angiogenesis.

It is well established that a rate-limiting step in solid tumor growth is the ability to recruit blood vessels from the host tissue (for a review, see ref. 1). Angiogenesis has become a major target for antitumor therapy on the premise that limiting angiogenesis will retard tumor growth and will inhibit metastatic spread of tumor cells.

The tumor "angiogenic switch" is determined by the net balance of angiogenic stimulators and natural inhibitors of (2, 3). VEGF is a potent angiogenic factor mediating developmental, physiological, and pathological neovascularization. In the context of tumor angiogenesis, VEGF expression is up-regulated as a consequence of oncogene activation or a loss of tumor suppressor (4–8). In addition to a genetic angiogenic switch, VEGF expression is induced in tumors by hypoxia and/or hypoglycemia generated whenever the angiogenic response is insufficient and vascular growth is lagging behind tumor growth (9–11). Augmented VEGF expression is correlated with increased tumor growth and vascularity (12, 13) and the inhibition of VEGF production or function leads to inhibition of tumor growth (14–17). Thus, it appears that VEGF plays a key role in the promotion of tumor angiogenesis. In the present study we examined whether the level of VEGF produced by tumor cells may determine the size and shapes of

tumor vessels. In particular, we examined the thesis that excessive production of VEGF may lead to formation of hyperfused vessels, such as those found in hemangioblastomas.

Growth factors, in general, may also function as survival factors for the respective target cell (18, 19). We have recently shown that VEGF is required for the maintenance of newly formed blood vessels in a natural developmental setting of retina neovascularization (20). Importantly, dependence on VEGF for survival is transient and, upon their maturation, vessels switch to a VEGF-independent state. Tumor expansion is associated with a continuous formation of new vessels and remodeling of existing vessels. We reasoned that these vessels may be sensitive to loss of VEGF resulting in regression of the existing tumor vasculature. To test this hypothesis, we constructed an inducible VEGF expression system in a xenografted C6 glioma tumor. We show that regression of preformed tumor vessels can be induced by VEGF withdrawal. The finding that VEGF is required for the maintenance of immature/remodeling tumor vessels may be exploited to increase the efficiency of anti-angiogenesis tumor therapy.

METHODS

Preparation and Analysis of pTET-VEGF Cell Lines. pTET-VEGF was constructed using the full-length coding sequence of mouse VEGF₁₆₅ amplified by PCR from a VEGF cDNA clone with the following primers and cloned as a *HindIII*–*EcoRV* fragment into pTET-Splice (BRL): (5' primer, 5'-CGCGAAGCTTCCACCATGGACTTTCTGCTCTC-TTGGGT-3'; 3' primer, 5'-CGCGGATATCACCGCCTTG-GCTTGTGTCACA-3'). C6 glioma cells were cotransfected with pTET-VEGF, pTET-TAK (BRL), and a plasmid encoding G418 resistance. Following stable selection in G418, colonies were picked, amplified, and tested for a tetracycline-regulated expression of VEGF as follows. Cells were grown for 48 h in the presence or absence of 1 µg/ml tetracycline. Total RNA (2 µg) from these cells was subjected to a reverse transcriptase–PCR analysis using random hexamers as reverse transcriptase primers and the VEGF-specific primers described above for PCR amplification. To serve as internal standard, ribosomal protein L19-specific primers (5'-CTGAAGGT-CAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3') were included in the PCR. Thirty amplification cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) were used. The resulting products were visualized by electrophoresis through 2% agarose in the presence of ethidium bromide. Approximately half of the VEGF-expressing colonies showed a tetracycline-regulated expression. Analysis of the particular clone chosen for further study is shown in Fig. 1.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1997 by The National Academy of Sciences 0027-8424/97/948761-6\$2.00/0
PNAS is available online at <http://www.pnas.org>.

Abbreviations: VEGF, vascular endothelial growth factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

Inoculation of Nude Mice. Cells (5×10^5) were injected subcutaneously into Nude mice (Harlan). Mice were given tetracycline when tumors reached a size of 0.5–1 cm². Tetracycline was used at a concentration of 100 µg/ml with 5% sucrose, and the drinking water changed every other day. Tumors were resected into buffered formalin and embedding in paraffin.

Histochemistry. Sections (4 µm) were cut from paraffin blocks and placed onto precoated slides (Sigma). Slides were deparaffinized in two changes of xylene (10 min) and rehydrated gradually from 100% ethanol to PBS. Hematoxylin and eosin staining was used to examine hemorrhage and necrosis. Blood vessel endothelial cells were visualized either by incubation with the *Bandeira simplicifolia* isolectin B4 or anti-von Willebrand factor. Lectin staining was done in PBS (biotin-conjugated; Sigma) overnight, followed by incubation with extra-avidin-peroxidase (1:20 dilution; BioMakor, Rehovot, Israel). Anti-von Willebrand factor was used 1:1,000 following 20-min trypsinization (0.1% at 37°C) and blocking with 10% goat serum/1% BSA. The primary antibody was followed by anti-rabbit horseradish peroxidase, and both lectin and antibodies were visualized with 3-amino-9-ethylcabazole (AEC; Sigma) in 50 mM Tris (pH 5.0).

RESULTS

A System for Switchable VEGF Expression in Xenografted Tumors. To determine the effects of VEGF induction and, in turn, withdrawal during tumor neovascularization, a switchable VEGF expression system was created. In the “Tet-off” expression system employed, expression of VEGF by tumor cells is repressed when tetracycline is added to the drinking water of tumor-bearing animals and is induced upon withdrawal of tetracycline.

C6 glioma cells were cotransfected with a VEGF₁₆₅ encoding cDNA driven by a tetracycline-responsive cytomegalovirus promoter and a vector encoding a transactivator protein that will activate VEGF expression only in the absence of tetracycline. To secure a nonleaky expression of the target gene at the “off” state, the transactivator protein itself is negatively regulated by tetracycline (see *Methods* for details). Stably transfected colonies in which VEGF expression is tightly regulated by tetracycline were selected. Fig. 1 *Left* shows an example of a C6 subline that expressed high levels of VEGF in the absence of tetracycline but barely detectable VEGF when tetracycline was included in the culture medium. This particular subline (designated C6 pTET-VEGF) was chosen for inoculation in Nude mice.

Over-Expression of VEGF Results in Tumor Hypervascularity and a Hemangioblastoma-Like Phenotype. C6 glioma cells produce well-vascularized tumors when inoculated in

mice. These tumors express a fairly high level of VEGF in a constitutive manner and production of VEGF is further augmented in hypoxic regions of the tumor (9–11, 21, 22). Despite a strong angiogenic response, the rapid increase in tumor mass is not matched by sufficient vascular growth, and extensive areas of necrosis develop in C6 glioma tumors grown under the skin of nude mice (Fig. 1*A*).

When C6 pTET-VEGF cells were inoculated subcutaneously and allowed to grow while VEGF expression is switched on, three phenotypic differences were reproducibly detected (compare Fig. 1*A* with *B*): (i) a highly significant increase in vascular density was observed, (ii) tumor necrosis was greatly reduced, and (iii) abnormally large and irregularly shaped tumor vessels and vascular sacs were formed. The first two differences suggested that a suboptimal production of a positive regulator like VEGF might be rate-limiting in neovascularization of gliomas. The results also showed that tumor necrosis in this tumor is a consequence of an insufficient angiogenic response. Of particular interest was the finding that over-production of VEGF leads to exceptionally large vessel diameter and vascular sacs. Examination of serial sections suggested that these structures were sometimes due to fusion of multiple blood vessels (data not shown). The striking resemblance to vascular structures found in hemangioblastoma supports the notion that over-expression of VEGF plays an important role in development of hemangioblastomas (7, 23, 24).

Withdrawal of VEGF Results in Regression of Newly Formed Tumor Vessels. To examine the maintenance requirement for VEGF on tumor vessels, tumors were first allowed to grow in the absence of tetracycline (switch “on”) until reaching a size of 0.5–1 cm². At this stage of tumor expansion blood vessels are intact (Fig. 2*A*). Tetracycline was then added to the drinking water to shut off VEGF expression, and tumors were resected from a single tumor-bearing mice at 24-h intervals. Hemorrhaging foci were detected as early as 24 h from VEGF withdrawal (Fig. 2*B*). The severity of hemorrhaging increased during the next 2 days and was evident as extensive areas occupied by erythrocyte escapees not contained in blood vessels (Fig. 2*C* and *D*). A marked decrease in the number of intact vessels was observed in these regions during the first 3 days from VEGF withdrawal. At later time points, extensive areas of the tumor underwent necrosis (Fig. 2*D* and *E*). This chain of events was highly reproducible and was independent of tumor size at the time of VEGF withdrawal. We conclude that VEGF is required for maintenance of at least a fraction of tumor vessels, and that its abrupt withdrawal during neovascularization results in regression of existing vessels. Interestingly, not all tumor vessels were effected by VEGF withdrawal. This is illustrated in Fig. 2*F*, which shows a tumor resected 5 days after switching off VEGF expression. A region

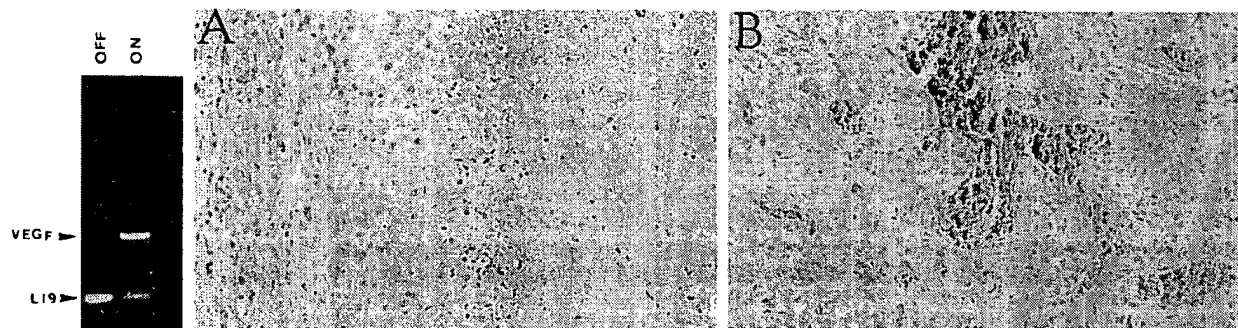


FIG. 1. The effect of VEGF₁₆₅ over-expression on tumor and vessel growth. (*Left*) Reverse transcriptase-PCR analysis of VEGF₁₆₅ mRNA in a C6 pTET-VEGF clone grown in the presence (“off”) and absence (“on”) of tetracycline. Arrowheads point at coamplified fragments of VEGF₁₆₅ and L19 ribosomal protein. (*Right*) Hematoxylin and eosin staining of sections from C6 tumors grown in Nude mice either in the absence (*A*) or presence (*B*) of tetracycline. Note particularly the extensive necrosis in *A* (to the right of the figure) and the abnormal large vessels in *B*.

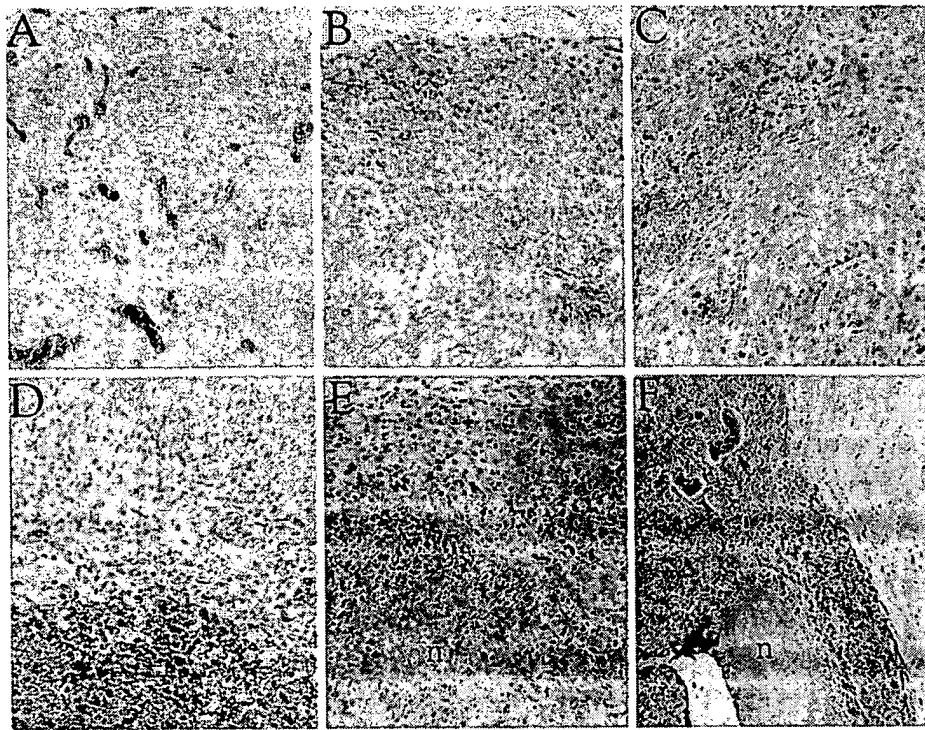


FIG. 2. Vessel regression and tumor necrosis following shut off of VEGF expression. Hematoxylin and eosin staining of sections from C6 tumors grown in Nude mice for 2 weeks in the absence of tetracycline and resected 0 h (A), 24 h (B), 48 h (C), 72 h (D), 4 days (E), and 5 days (F) after administration of tetracycline to shut off expression of VEGF from the transfected VEGF₁₆₅. n, Necrosis.

is highlighted to show side-by-side where vessel regression has led to necrosis yet adjacent tissue contains a healthy tumor vasculature.

Regression of Tumor Vessels Involves Endothelial Cell Detachment. To gain insights into the mechanism of blood

vessel regression, endothelial cells were localized by *in situ* immunostaining during early times following administration of tetracycline. As shown in Fig. 3, endothelial cells were observed in the process of detachment. Progressive stages in this process are shown. Fig. 3A shows endothelial cells separating

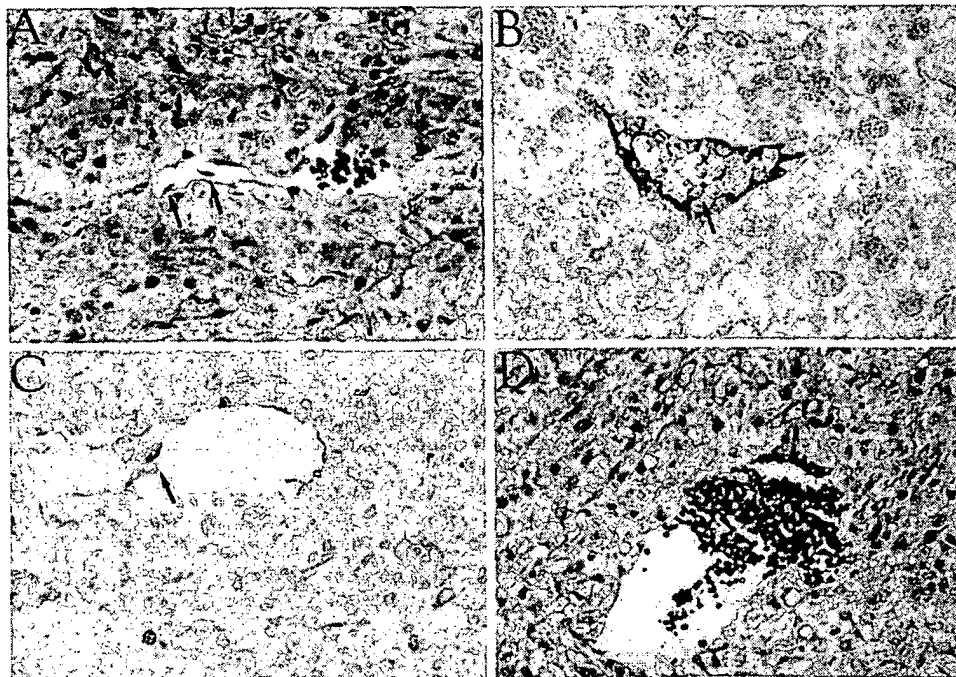


FIG. 3. Endothelial cell detachment and erythrocyte escape from blood vessels. (A–D) Sections from a tumor resected 24 h after VEGF shut off. A and D were stained with hematoxylin and eosin. Endothelial cells were visualized in B by staining with anti-von Willebrand antibodies and in C by staining with *B. simplicifolia* isolectin B4. Arrows point at endothelial cells being shed into the lumen.

from the vessel wall (highlighted by arrows). Fig. 3B shows a still-continuous endothelial cell lining (stained with anti-von Willebrand factor antibodies) with only a single endothelial cell on its way to being shed into the lumen. Fig. 3C shows detachment of the few remaining endothelial cells (stained with *B. simplicifolia* isolectin B4). Fig. 3D shows erythrocytes escaping from the lumen of a vessel devoid of endothelial cell lining.

It is well established that cells that lose contact with their extracellular matrix undergo apoptosis. Such a phenomenon has been specifically observed with endothelial cells (25). To observe endothelial cell apoptosis, DNA fragmentation products were stained by *in situ* 3' end labeling (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; TUNEL). Occasionally, TUNEL-positive cells were observed in the endothelial cell lining of blood vessels (Fig. 4 *Left*). However, the majority of TUNEL-positive cells were seen as isolated cells that could not be identified as endothelial cells simply on a contextual basis. We confirmed that isolated TUNEL-positive cells were in fact endothelial cells using double-labeling with TUNEL and anti-von Willebrand factor antibodies (Fig. 4 *Center and Right*). These findings supported the hypothesis that detachment of the endothelium was the primary effect of VEGF-loss and preceded endothelial cell apoptosis.

DISCUSSION

We have devised an experimental system that allows to modulate the level of VEGF production during progressive stages of tumor growth and neovascularization. This system enabled us to determine, on the one hand, the effects of VEGF over-production and, on the other hand, the consequences of untimely withdrawal of VEGF. This experimental system is particularly suitable for simulating a situation of a fluctuating VEGF expression, a condition that is likely to develop due to fluctuating tumor oxygenation (26, 27) and a likely cause of vascular injury.

Vascular and Tumor Phenotypes Resulting from Over-Expression of VEGF. With the contribution of VEGF produced from the transfected VEGF₁₆₅ expression plasmid, blood vessels grew to a significantly higher density than in control tumors expressing only the endogenous VEGF, suggesting that neovascularization in this system is limited by the availability of angiogenic factors. This result is consistent with previous findings showing that increased expression of VEGF in a xenografted carcinoma tumor also results in increased vascularity (12, 28).

Progression from benign astrocytoma to malignant glioblastoma multiforme is associated with a progressive increase in tumor vascularity. Yet, even the most vascularized glioblastomas are characterized by extensive necrosis. Our finding that boosting VEGF production results in tumor expansion without tumor necrosis again suggests that tumor neovascularization is unable to match the rapid growth of this tumor due to insufficient production of a positive regulator of angiogenesis.

Perhaps the most dramatic phenotype associated with over-expression of VEGF is the formation of abnormally large and malformed vessels. A larger vessel diameter could be, in principle, the result of either dilation, circumferential growth, or vascular fusions. Judging from the course of individual vessels followed in serial sections (data not shown), as well as from their irregular circumference (e.g., Fig. 1B), we favor the third possibility, namely that fusion of vessels occurred at multiple points of contact with neighboring vessels. Thus, it is likely that vascular fusions will be pronounced mostly in cases of an exceedingly high vascular density. VEGF was found to induce a similar hyperfused vessel phenotype when injected during development of quail embryos (29). Thus, in both embryonic and tumor development the level of VEGF produced at the site of neovascularization seems to effect vessel diameter. The vascular pattern shown in Fig. 1, particularly the formation of vascular sacs, resembles vascular structures found in hemangioblastomas. On the basis of findings that hemangioblastomas express relatively high levels of VEGF, it has been suggested that VEGF may mediate neovascularization and cyst formation in capillary hemangioblastoma (23, 24). Findings shown here support this suggestion.

Vascular Regression Resulting from Withdrawal of VEGF. A major finding of this study is that by switching off expression from the transfected VEGF expression plasmid, preformed tumor vessels regress, suggesting that VEGF functions as a survival factor for tumor vessels. Certain vascular networks regress normally during development (e.g., the hyoid vascular system of the eye regresses upon completion of lens development), and it is likely that a developmentally programmed regression of transient vascular networks is caused by down-regulation of an essential vascular survival factor. A distinction should be made, however, between regression of a fully matured, functional vascular system and vascular obliteration that is coupled to the process of neovascularization or remodeling. An example of the latter is the process of vascular pruning associated with retina neovascularization. We have recently shown that pruning of retina vessels is induced when VEGF is down-regulated to a level lower than the level required to sustain newly formed, immature vessels (20). We

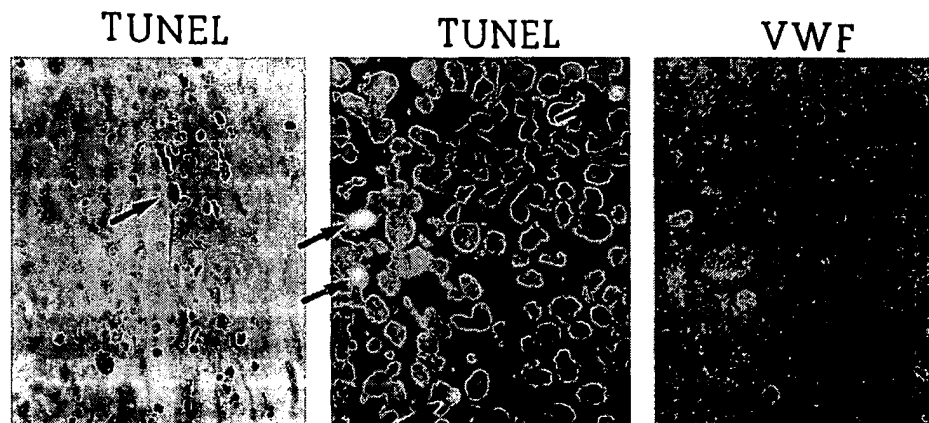


FIG. 4. Extra-vascular TUNEL-positive endothelial cells. (*Left*) TUNEL-positive endothelial cell (arrow) in the wall of a partially degenerated vessel, 24 h after VEGF shut off (counterstained with hematoxylin and eosin). (*Center and Right*) Double-labeling of a tumor section with TUNEL (using a fluorescein isothiocyanate-fluorophore) (*Center*) and anti-von Willebrand (VWF) antibody (using a rhodamine fluorophore) (*Right*).

argued that vascular pruning is an integral part of a process of adjusting the vascular density to match oxygen requirements of the surrounding tissue. VEGF, by virtue of its up-regulation by hypoxia and down-regulation by hyperoxia, is poised to mediate both vascular expansion as well as vascular regression. We reasoned, therefore, that the potential for regressing blood vessels under conditions of VEGF deprivation will be shared with neovascularizing tumors. The results shown in Figs. 2 and 3 indicate that this is indeed the case. It should be noted that, in the experimental system used, even when VEGF is "switched off" there is still protein produced from the endogenous alleles and yet many vessels regress. This suggests that there is a direct correlation between the amount of available VEGF and the number of vessels it can sustain.

From a mechanistic point of view, the shedding of endothelial cells from the vessel wall appeared to be an early step in vessel regression. Though we could occasionally detect TUNEL-positive endothelial cells in intact vessels, more often the endothelial cells had separated from the vessel wall before evidence of DNA fragmentation could be detected. A similar observation was recently made in the process of vessel regression in the corpus luteum (30). It is well established that cells that lose contact with their extracellular matrix undergo apoptosis (31). Such a phenomenon has been specifically observed with endothelial cells (25, 32). Findings reported here suggest that VEGF may function as a vascular survival factor by mediating interaction of endothelial cells with the underlying matrix. In turn, the transition to a VEGF-independent state may take place concomitantly with establishment of endothelial cell-extracellular matrix interactions that are independent of VEGF.

Clinical Implications of Vascular Regression Caused by VEGF Withdrawal. Should enforced regression of tumor vessels be considered as a component of anti-angiogenic cancer therapy? At the time of clinical diagnosis solid tumors are already well vascularized and contain vessels at different degrees of maturity. The success of survival-factor loss therapy in tumors might depend on the fraction of immature vascular elements engaged in neovascularization or remodeling and might, therefore, differ from one type of tumor to another (33, 34). Analysis of primary human glioblastomas has shown that the 5-bromodeoxyuridine (BrdUrd) labeling index of tumor endothelial cells is a 4.5%. Moreover, the labeling index of glomerular-shaped vessels, which are formed mostly in response to VEGF induced by environmental stress (9), is as high as 20% (35). Thus, it appears that ongoing neovascularization and remodeling will render a significant fraction of tumor vessels an appropriate target for enforced regression. Also, the collapse of a relatively small fraction of vessels might cause damage to a much larger fraction of tumor tissue. Our observations massive areas of the tumors were effected by hemorrhage and subsequently underwent necrosis (Fig. 2D and E) is consistent with this suggestion. The appearance of blood vessels and healthy tumor tissue after longer times (Fig. 2F) may be due to revascularization mediated by the endogenous VEGF or by other angiogenic factors, such as fibroblast growth factor produced by C6 cells (36). Alternatively, a fraction of the tumor vessels may be "mature" and refractory to VEGF loss. It is likely that the high efficiency of anti-VEGF treatments to reduce the size of xenografted tumors (14–16) is, at least in part, due to regression of preformed tumor vessels (37).

VEGF is currently being used in clinical trials to stimulate neovascularization (i.e., to induce formation of collateral blood vessels). Our studies suggest that, while VEGF may effectively induce endothelial cell proliferation, its untimely discontinuation might lead to subsequent regression of the new endothelium. For example, findings that a therapeutic neovascularization following VEGF gene transfer was temporary and failed to sustain circulation in a gangrenous leg after 2 months (38) could be explained by a requirement for a sustained VEGF production to maintain the newly formed

vessels. We feel, therefore, that our finding that newly formed or remodeling blood vessels require sustained VEGF levels will be critical in the success of many angiogenic and anti-angiogenic therapies.

We thank Ahuva Itin and Hadassah Gnessin for excellent technical assistance. This research was supported by the Israel Science Foundation (administered by the Israel Academy of Sciences) and by the Mireille and James I. Levy Foundation. L.E.B. was funded by the Valazzi-Pikovsky Fellowship Fund.

1. Hanahan, D. & Folkman, J. (1996) *Cell* **86**, 353–364.
2. Holmgren, L., O'Reilly, M. S. & Folkman, J. (1995) *Nat. Med.* **1**, 149–153.
3. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Cao, Y., Moses, M., Lane, W. S., Sage, E. H. & Folkman, J. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59**, 471–482.
4. Mukhopadhyay, D., Tsiokas, L. & Sukhatme, V. P. (1995) *Cancer Res.* **55**, 6161–6165.
5. Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T. & Kerbel, R. S. (1995) *Cancer Res.* **55**, 4575–4580.
6. Grugel, S., Finkenzeller, G., Weindel, K., Barleon, B. & Marme, D. (1995) *J. Biol. Chem.* **270**, 25915–25919.
7. Witzigmann Voos, S., Breier, G., Risau, W. & Plate, K. H. (1995) *Cancer Res.* **55**, 1358–1364.
8. Kieser, A., Weich, H. A., Brandner, G., Marme, D. & Kolch, W. (1994) *Oncogene* **9**, 963–969.
9. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992) *Nature (London)* **359**, 843–845.
10. Plate, K. H., Breier, G., Weich, H. A. & Risau, W. (1992) *Nature (London)* **359**, 845–848.
11. Shweiki, D., Neeman, M., Itin, A. & Keshet, E. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 768–772.
12. Zhang, H. T., Craft, P., Scott, P. A., Ziche, M., Weich, H. A., Harris, A. L. & Bicknell, R. (1995) *J. Natl. Cancer Inst.* **87**, 213–219.
13. Takahashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R. & Ellis, L. M. (1995) *Cancer Res.* **55**, 3964–3968.
14. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993) *Nature (London)* **362**, 841–844.
15. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W. & Ullrich, A. (1994) *Nature (London)* **367**, 576–579.
16. Millauer, B., Longhi, M. P., Plate, K. H., Shawver, L. K., Risau, W., Ullrich, A. & Strawn, L. M. (1996) *Cancer Res.* **56**, 1615–1620.
17. Borgstrom, P., Hillan, K. J., Sriramarao, P. & Ferrara, N. (1996) *Cancer Res.* **56**, 4032–4039.
18. Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. & Jacobson, M. D. (1993) *Science* **262**, 695–700.
19. Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. & Jacobson, M. D. (1994) *Philos. Trans. R. Soc. London B* **345**, 265–268.
20. Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J. & Keshet, E. (1995) *Nat. Med.* **1**, 1024–1028.
21. Plate, K. H., Breier, G., Millauer, B., Ullrich, A. & Risau, W. (1993) *Cancer Res.* **53**, 5822–5827.
22. Stein, I., Neeman, M., Shweiki, D., Itin, A. & Keshet, E. (1995) *Mol. Cell. Biol.* **15**, 5363–5368.
23. Morii, K., Tanaka, R., Washiyama, K., Kumanishi, T. & Kuwano, R. (1993) *Biochem. Biophys. Res. Commun.* **194**, 749–755.
24. Bohling, T., Hatva, E., Kujala, M., Claesson Welsh, L., Alitalo, K. & Haltia, M. (1996) *J. Neuropathol. Exp. Neurol.* **55**, 522–527.
25. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancione, L., Dejana, E. & Colotta, F. (1994) *J. Cell Biol.* **127**, 537–546.
26. Trotter, M. J., Chaplin, D. J., Durand, R. E. & Olive, P. L. (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 931–945.
27. Chaplin, D. J., Olive, P. L. & Durand, R. E. (1987) *Cancer Res.* **47**, 597–601.
28. Fox, S. B., Gatter, K. C., Bicknell, R., Going, J. J., Stanton, P., Cooke, T. G. & Harris, A. L. (1993) *Cancer Res.* **53**, 4161–4163.
29. Drake, C. J. & Little, C. D. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7657–7661.
30. Modlich, U., Kaup, F.-J. & Augustin, H. G. (1996) *Lab. Invest.* **74**, 771–780.
31. Merdith, J. E. & Schwarz, M. A. (1997) *Trends Cell Biol.* **7**, 146–150.

32. Merdith, J. E., Fazeli, B. & Schwartz, M. A. (1993) *Mol. Biol. Cell.* **4**, 953–961.
33. Rak, J. & Kerbel, R. S. (1996) *Cancer Metastasis Rev.* **15**, 231–236.
34. Barinaga, M. (1997) *Science* **275**, 482–484.
35. Nagashima, T., Hoshino, T. & Cho, K. G. (1987) *Acta Neuropathol.* **73**, 301–305.
36. Powell, P. P. & Klagsbrun, M. (1993) *Exp. Cell Res.* **209**, 224–230.
37. Yuan, F., Chen, Y., Dellian, M., Safabakhsh, N., Ferrara, N. & Jain, R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14765–14770.
38. Isner, J. M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K. & Symes, J. F. (1996) *Lancet* **348**, 370–374.

LEE M. ELLIS, MD
Professor
Departments of Surgical Oncology
and Cancer Biology

WENBIAO LIU, MD
Coordinator
Research Laboratory
FAN FAN, MD
Research Assistant
YOUNG DO JUNG, MD
NIELS REINMUTH, MD
OLIVER STOELTZING, MD
AKIHIKO TAKEDA, MD
MORIHISA AKAGI, MD
Research Fellows
Department of Cancer Biology

ALEXANDER A. PARIKH, MD
Surgical Fellow
SYED AHMAD, MD
Oncology Fellow
Department of Surgical Oncology
The University of Texas
M. D. Anderson Cancer Center
Houston, Texas

By definition, angiogenesis is the establishment of a neovascular blood supply derived from preexisting blood vessels, whereas vasculogenesis is the embryonic establishment of a blood supply from mesodermal precursors such as angioblasts or hemangioblasts. Tumor angiogenesis more accurately refers to a combination of angiogenesis and vasculogenesis in which the main blood supply to a tumor is derived from preexisting blood vessels, although circulating endothelial cell precursors may contribute to the growing endothelial cell mass.

Numerous investigators have established the association of tumor angiogenesis with metastasis.[1] Indeed, it is thought that tumor angiogenesis is essential for the growth of both primary and metastatic tumors,[2,3] and provides both nutri-

Synopsis of Angiogenesis Inhibitors in Oncology

ABSTRACT

Angiogenesis is a dynamic process essential for primary tumor growth and metastases. New insights into the basic understanding of the biologic processes responsible for angiogenesis have led to the characterization of potential therapeutic targets. Several strategies for the development of antiangiogenic therapeutic modalities have been employed, including agents that (1) decrease the activity of specific angiogenic factors, (2) decrease the activity of endothelial survival factors, (3) increase the activity of naturally occurring antiangiogenic agents, or (4) indirectly downregulate angiogenic and survival factor activity. Because antiangiogenic therapy is unlikely to induce tumor regression, the criteria for efficacy must be evaluated by means other than the standard response criteria used to evaluate cytotoxic chemotherapy. Further, the redundancy of molecules responsible for the angiogenic process suggests it is unlikely that a single antiangiogenic agent will provide prolonged inhibition of angiogenesis. Nevertheless, the understanding of the basic principles that drive tumor angiogenesis will lead to the development of therapies that will likely prolong survival without the toxicity associated with standard chemotherapy.

ents and oxygen to the growing tumor mass. A neovascular blood supply is also essential for increasing the chance that tumor cells will gain access to the circulation and subsequently begin the process of forming metastases at different sites. Once a tumor establishes an invasive phenotype in the organ of metastasis, it must then establish its own neovascular blood supply in order to grow.

This process, more complex than was previously thought, requires the

coordinated activities of multiple factors and cell types. For tumors to develop a neovascular blood supply, tumor and host cells must secrete proangiogenic factors that offset the activities of inhibitory angiogenic factors. In addition, the newly derived tumor endothelium must respond to and survive in a relatively caustic microenvironment; thus, endothelial cell-survival factors are essential in the maintenance of this neovascular structure. Nevertheless, because the pro-

primary tumor understanding of have led to the al strategies for ilities have been tivity of specific othelial survival ; antiangiogenic d survival factor to induce tumor l by means other cytotoxic chemo- ponsible for the e antiangiogenic sis. Nevertheless, nor angiogenesis ll likely prolong d chemotherapy.

vities of multiple fac- types. For tumors, o ascular blood supply, t cells must secrete factors that offset the bitory angiogenic fac- n, the newly derived um must respond a relatively causa ent; thus, endothelial actors are essential ce of this neovascu- less, because the pro-

cess of angiogenesis is regulated by redundant factors and pathways, inhibition of any single pathway is unlikely to lead to prolonged response in most patients with solid malignancies.

More than 1,700 papers were published on aspects of tumor angiogenesis in 2001. This field of research is closely scrutinized by scientists, clinicians, patients, and the media. However, data from phase I and II antiangiogenic trials have only been reported in abstract form; most of the data is too preliminary to draw meaningful conclusions. Further, phase III trials, even if they have reached their target accrual, are several years away from maturity with appropriate follow-up. The published reports available on clinical trials have thus far produced little more than information on the toxicity and tolerability of angiogenesis inhibitors.

Given the complexity of angiogenesis, the basic biology of this process must be better understood before effective antiangiogenic therapy can be developed. Herein, we review recent advances in the basic understanding of angiogenesis and the role of angiogenic factors in tumorigenesis. Further, we will discuss overall strategies, expectations, and future directions of antiangiogenesis therapy.

The Angiogenic Switch in Tumor Progression

Under normal physiologic conditions, the activity of endogenous pro-angiogenic factors equals that of antiangiogenic factors, leading to a homeostatic balance that prevents the uncontrolled growth of tissues. Pathologic angiogenesis occurs when the effect of stimulatory molecules outweighs the effect of inhibitory molecules (Table 1).^[4] Intensive study of the angiogenic process led to the realization that this process involves more than simple proliferation of endothelial cells. This process also requires endothelial cells to divide, invade the basement membrane, migrate, and undergo differentiation and capillary-tube formation (Figure 1).^[4] This process is driven not only by angiogenic molecules, but also by other factors, such as degradative en-

Table 1

Endogenous Regulators of Angiogenesis

Stimulatory Angiogenic Factors	Inhibitory Angiogenic Factors
Acidic fibroblast growth factor	Angiostatin
Angiogenin	Antithrombin III fragment
Basic fibroblast growth factor	Endostatin
Fibroblast growth factors -3, -4	Fragment of platelet factor-4
Hepatocyte growth factor/scatter factor	Interferon -alpha, -beta
Interleukin-8	Interferon-inducible protein-10
Placental growth factor	Maspin
Platelet-derived growth factor	METH-1, -2
Pleiotropin	Prolactin fragment
Proliferin	Thrombospondin-1, -2
Thymidine phosphorylase/platelet-derived endothelial-cell growth factor	Tumstatin
Transforming growth factor -alpha, -beta	Vascular endothelial growth inhibitor
Tumor necrosis factor-alpha	Vasostatin
Vascular endothelial growth factor/vascular permeability factor	

zymes, that mediate the above processes. Interestingly, the processes of tumor angiogenesis (as noted above) and the processes of tumor-cell invasion are very similar.

Vascular Endothelial Growth Factor

The best characterized of the stimulatory angiogenic factors is vascular endothelial growth factor (VEGF), which has also been associated with an aggressive phenotype in numerous solid malignancies.^[5-10] Vascular endothelial growth factor is a 32- to 44-kDa protein secreted by nearly all cells.^[4] At least four isoforms of VEGF, derived from alternate splicing of the mRNA, have been characterized.^[4,11] The smaller isoforms, VEGF-121 and VEGF-165 (the numbers denote the number of amino acids), are secreted from cells. The larger isoforms, VEGF-189 and VEGF-205, are cell associated, and their functions are currently being investigated.

One distinguishing factor of VEGF

is its ability to induce vascular permeability. In fact, this factor was originally named vascular permeability factor (VPF) and was subsequently found to be homologous to VEGF.^[12-14] The extent of vascular permeability induced by VEGF is 50,000 times that of histamine, which was historically the gold standard for induction of permeability. This action by VEGF allows proteins to diffuse into the interstitium and to form the lattice network onto which endothelial cells migrate.

In the past, it was believed that receptors for VEGF were expressed predominantly on endothelial cells. Recently, the VEGF receptors have also been found on cells of neural origin, Kaposi's sarcoma cells, hematopoietic precursor cells, certain leukemias, and selected epithelial tumors.^[15,16] The current nomenclature for the three known VEGF receptors is VEGFR-1(Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). These tyrosine kinase receptors require dimerization.

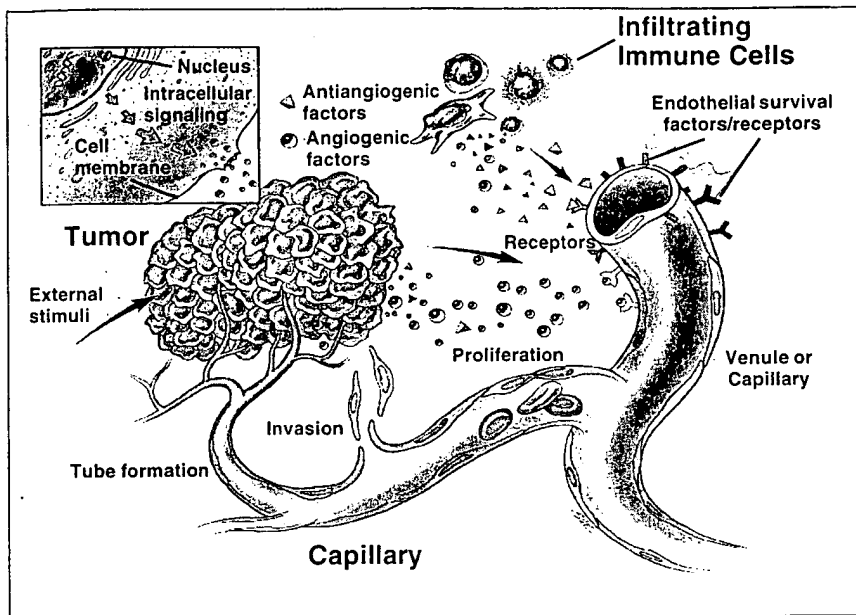


Figure 1: The Angiogenic Process—Tumor angiogenesis is a series of linked and sequential steps culminating in the development of a neovascular blood supply to a tumor mass. The proangiogenic process is balanced by the activity of the antiangiogenic molecules that are necessary for homeostasis. New blood vessels form when the activity of the proangiogenic molecules exceeds that of the antiangiogenic molecules. Although proangiogenic factors can be constitutively expressed, their expression can be increased by certain stimuli such as hypoxia, low pH, cytokines, growth factors, tumor size, activated oncogenes, signal transduction pathways, or loss of tumor suppressor gene function. Reproduced with permission from Fidler et al.[4]

to induce intracellular signaling following specific ligand binding. The receptors for VEGF may mediate distinct functions within the endothelial cell. For example, VEGFR-1 may be important in migration, whereas VEGFR-2 may be important in the induction of permeability, endothelial cell proliferation, and survival. Neuropilin, a receptor involved in neuronal guidance, has been identified as a coreceptor for VEGF-165 and may enhance angiogenesis.

Recently, the angiopoietin family of ligands has been found to play an important role in the homeostasis of the tumor vasculature. The angiopoietins are proteins involved in angiogenesis that bind to the endothelial-cell-specific tyrosine kinase receptor Tie-2. Angiopoietin-1 (Ang-1) acts as an agonist and is involved in endothelial-cell differentiation and stabilization.[17] In contrast, Ang-2 binds to Tie-2 and blocks the binding of Ang-1 to this

receptor.[18,19] This blockade leads to endothelial-cell destabilization and vascular regression.[20]

Angiogenesis Hypotheses

It has been hypothesized that tumor angiogenesis involves the co-option of preexisting blood vessels in addition to vascular regression and subsequent neovascularization.[20] This theory suggests that tumors initially co-opt existing blood vessels within an organ for their nutrient blood supply. Shortly thereafter, the existing vasculature becomes destabilized, most likely through the release of Ang-2 by endothelial cells. This loss of vascular integrity leads to relative hypoxia within the tumor, which in turn leads to upregulation of VEGF in the tumor cells. These events then lead to a robust angiogenic response. At that stage, the newly developed endothelial cells require stabilization, which is achieved through release of Ang-1 by endo-

thelial cells and possibly through continued response to VEGF. Thus, the process of angiogenesis depends on the temporal coordination of factors that regulate pathways in the establishment of stable conduits that provide a nutrient blood supply to the tumor.

In vitro, Ang-1 has been shown to be angiogenic, inducing tube formation of endothelial cells growing on extracellular matrix components. However, recent in vivo studies have demonstrated that Ang-1 may in fact be antiangiogenic. We have shown that overexpression of Ang-1 in human colon cancer cells leads to decreased angiogenesis and tumor growth, whereas overexpression of Ang-2 leads to an increase in tumor growth and angiogenesis.[21] This finding is consistent with immunohistochemical studies that demonstrate that colon cancers express Ang-2 but do not express Ang-1. This suggests that the imbalance of Ang-2 over Ang-1 may be an initiating factor in tumor angiogenesis. Others have also confirmed the above findings in breast and gastric cancer tumor cells and cell lines.[22,23]

Numerous nonspecific angiogenic factors affect the growth of cell types other than endothelial cells. These factors include the fibroblast growth factors (acidic and basic), transforming growth factor- α , and epidermal growth factor (EGF), both of which bind to the EGF receptor; platelet-derived growth factor (PDGF); platelet-derived endothelial-cell growth factor (PD-ECGF); angiogenin; and the CXC chemokines interleukin-8, macrophage inflammatory protein 1, platelet factor 4, and growth-related oncogene alpha (Table 1).[24]

These factors are known to be angiogenic in in vivo models but are not specific for endothelial cells. However, as noted earlier, a single molecule or family of molecules does not drive angiogenesis; rather it depends on the cooperation and integration of various factors leading to endothelial cell proliferation, migration, invasion, differentiation, and capillary-tube formation. It has yet to be determined whether inhibiting

ibly through con-
VEGF. Thus, the
esis depends on
nation of factors
ays in the estab-
onduits that pro-
od supply to the

as been shown to
icing tube forma-
cells growing on
ix components,
vivo studies have
Ang-1 may in fact
We have shown
of Ang-1 in hu-
cells leads to de-
esis and tumor
overexpression of
increase in tumor
genesis.[21] This
nt with immuno-
lies that demon-
cancers express
press Ang-1. This
balance of Ang-2
an initiating fac-
ogenesis. Others
d the above find-
gastric cancer tu-
lines.[22,23]
pecific angiogen-
ie growth of cell
endothelial cells.
ude the fibroblast
cidic and basic),
h factor-alpha, and
factor (EGF), both
the EGF receptor,
growth factor
erived endothelial-
(PD-ECGF): an-
CXC chemokines
rophage inflamma-
telet factor 4, and
eogene alpha (Ta-

re known to be an-
vo models but are
endothelial cells.
d earlier, a single
of molecules does
nesis; rather it de-
peration and inte-
factors leading to
roliferation, migra-
fferentiation, and
mation. It has yet
whether inhibiting

the activity of a single angiogenic factor will lead to vascular compromise of significant duration. More likely, the redundancy in the angiogenic process will select for other angiogenic factors when a specific angiogenic factor is targeted.

Upstream Regulation of Angiogenic Factor Expression in Tumors

Tumors may constitutively express high levels of angiogenic factors or may express high levels of angiogenic factors in response to the tumor microenvironment. Signals that upregulate angiogenic factors include extracellular signals, intrinsic upregulation of signal transduction activity, and loss of tumor suppressor genes (Table 2).

Extracellular Signals

Extracellular signals that lead to the induction of angiogenic factor expression include environmental stimuli such as hypoxia or a decrease in pH.[25-27] In fact, hypoxia is the most potent stimulus for inducing angiogenic factors, especially VEGF. Hypoxic induction of VEGF is probably mediated through Src kinase activity, which then leads to downstream induction of signaling cascades and eventually to an increase in the activity of hypoxia-inducible factor-1 (HIF-1) alpha.[28,29] This factor then increases the transcription of the VEGF gene, which in turn leads to the induction of angiogenesis. Recent evidence suggests that activation of growth factor receptors may also increase HIF-1 alpha activity.[30]

Cyclooxygenase-2 is an enzyme constitutively overexpressed in colon cancer and other solid malignancies.[31] Its overexpression may play a role in malignant cell survival. In addition, elegant studies from Dubois and others have demonstrated that COX-2 can regulate VEGF expression and angiogenesis.[31-33] Thus, COX-2 inhibitors may provide a means of indirectly inhibiting angiogenesis with minimal toxicity.

Several studies have shown that activation of the EGF receptor (EGF-

R) can lead to induction of angiogenic factors in tumor cells.[34-36] In orthotopic models of bladder and pancreatic cancers, treatment of mice with an anti-EGF-R antibody led to a decrease in VEGF and interleukin-8 expression that was associated with a decrease in tumor growth and vascularity.[36,37] Other cytokines and growth factors such as insulin growth factors (IGF)-I and -II, hepatocyte growth factor, interleukin-1, and platelet-derived growth factor have all been shown to upregulate VEGF. Thus, antiangiogenic therapy could involve downregulation of upstream mediators of the angiogenic factors rather than targeting the angiogenic factors themselves.[28,38]

Intrinsic Upregulation of Signal Transduction

Once a growth factor or a cytokine binds to its receptor, a cascade of intracellular signaling events is initiated. Two specific signal transduction pathways are well known to mediate the upregulation of angiogenic factors: the phosphatidylinositol 3 (PI3)-kinase/Akt signal transduction pathway, which eventually leads to stabilization of HIF-1 alpha.[39,40] and the mitogen-activated protein kinase (MAPK) pathway, in which phosphorylation of Erk-1/2 activates factors that increase transcription of the VEGF gene.[41] Activated *ras* and *Src* have also been shown in in vivo models to be associated with increased VEGF production and angiogenesis.[42] Again, therapeutic strategies that target the upstream effector molecules in angiogenesis may be a rational means of preventing angiogenesis. Indeed, inhibitors of signal transduction molecules have been shown to inhibit angiogenesis in in vivo tumor models.[28]

Loss of Tumor Suppressor Genes

Protein products of tumor suppressor genes such as the von Hippel-Lindau (VHL) or p53 genes also regulate angiogenesis. The wild-type VHL protein represses transcriptional regulation of the VEGF gene by facilitating degradation of HIF-1.[43-45] A loss of heterozygosity with a

Table 2

Upstream Regulators of Angiogenic Factors That May Serve as Potential Targets for Therapy

Cyclooxygenase-2
Epidermal growth factor receptor/Her2/neu
Hypoxia-inducible factor-1 alpha
Insulin-like growth factor receptor-type I
Interleukin-1 alpha receptor
Oncogenes (<i>Src</i> , <i>ras</i> , <i>raf</i>)
Platelet-derived growth factor receptor
Tumor suppressor genes (p53, VHL)

VHL = von Hippel-Lindau.

mutation in the remaining VHL allele leads to loss of transcriptional control of the VEGF gene and overexpression of VEGF. Mutant p53 has also been associated with an increase in angiogenesis.[46] Reinsertion of the wild-type p53 gene into cells with mutant p53 can downregulate VEGF expression and angiogenesis. Thus, the process of angiogenesis is driven by external forces (including environmental stimuli), aberrations in internal signaling, and alterations in tumor suppressor gene function.

Effective Antiangiogenic Therapy

Overall Expectations

The knowledge that angiogenesis is essential for tumor growth and the formation of metastases has led to a large research effort in an attempt to discover effective antiangiogenesis compounds. However, angiogenesis not only is a pathologic process but

One or two copies of this article for personal or internal use may be made at no charge. Copies beyond that number require that a 9¢ per page per copy fee be paid to the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01970. Specify ISSN 0890-9091. For further information, contact the CCC at 508-750-8400. Write publisher for bulk quantities.

also is essential for homeostasis. Physiologic angiogenesis is important in reproduction, wound healing, and menses, as well as a compensatory response to ischemia in coronary-artery and peripheral vascular diseases. Thus, therapeutic efficacy of antiangiogenic therapy requires a balance where angiogenesis in tumors is inhibited without disrupting physiologic angiogenesis.

For example, controversy exists regarding the effects of antiangiogenic therapy and wound healing.[47-50] Because of the need for neovascularization in wound healing, one would expect that an effective antiangiogenic agent would inhibit healing similar to its antiangiogenic effect on tumor growth. However, treatment with endostatin did not significantly decrease the breaking strength of cutaneous wounds in mice,[49] and although it decreased functional blood vessels and matrix density in granulation tissue in another mouse model, endostatin did not significantly affect overall wound healing.[50] Interestingly, wound angiogenesis is being used as a surrogate marker of drug activity.

In addition to potential effects of antiangiogenic therapy on homeostasis, duration of antiangiogenic therapy and criteria for efficacy are other issues to be considered. Because most antiangiogenic therapies are intended to decrease the development of new blood vessels, the traditional end points for tumor treatment success or failure must be redefined. For example, a desirable response for standard chemotherapy is a 50% decrease in the cross-sectional area of a tumor; however, the desired end point after antiangiogenic therapy might be inhibition of further tumor growth (ie, tumor stabilization or prolongation of time to progression). Thus, the criteria for the effectiveness of antiangiogenic therapy (whether in the clinic or in the laboratory) must be considered from a new perspective relative to conventional therapies.

Although some reports exist of tumor regression in experimental models of angiogenesis,[51,52] such findings are rare; the vast majority of studies in this field demonstrate that

antiangiogenic therapy leads to an inhibition of tumor growth rather than a regression of established tumors.[53,54] Therefore, the ability to appropriately interpret the results from experimental models is critical to ensure that extrapolations to the clinical setting are not fraught with unrealistic expectations.

For example, a typical growth curve for a subcutaneously implanted tumor may demonstrate that antiangiogenic therapy significantly decreases tumor growth rate. In this preclinical model, this "positive" result may lead to clinical trials of that same agent. In the clinic, however, inhibition of tumor growth can be interpreted as "progressive disease" and the therapy thus considered a failure, particularly if tumor-imaging studies are done at short intervals. Therefore, longer periods of antiangiogenic therapy administration may be required to fully characterize the efficacy of antiangiogenic therapy (assessed by the inhibition of tumor growth rate and reduced metastases) compared with chemotherapy (assessed by decreases in tumor size).

Effective antiangiogenic therapy will probably need to be delivered on a chronic basis. Chronic administration will require that the agent be delivered easily (perhaps by the oral route) and have few cumulative long-term effects. As previously noted, the effect of antiangiogenic therapy may require longer evaluation intervals. One must also consider that the goal of standard antiangiogenic therapy is intended to decrease blood vessel formation and prevent further tumor growth, not cause tumor regression. Therefore, uniform response criteria should be developed for determining the effectiveness of antiangiogenic therapy (eg, time to progression, survival, quality of life); these criteria will probably differ from current criteria for tumor response to cytotoxic agents that include reductions in tumor size.

Overall Strategies

Despite the simplified view that antiangiogenic therapy simply interferes with the blood supply to a tu-

mor, the strategies in the development of antiangiogenic therapies are quite diverse and distinct. Antiangiogenic strategies can be classified under four major categories: (1) those that decrease the activity of specific angiogenic factors; (2) those that decrease the activity of endothelial survival factors; (3) those that increase the activity of naturally occurring antiangiogenic agents, such as angiostatin, endostatin, thrombospondin; and (4) those that indirectly downregulate activity of angiogenic and survival factors.

Decreased Activity of Angiogenic Factors

In the following discussion, VEGF will be the prototype molecule used to describe strategies to decrease the activity of angiogenic factors because it has been linked to the angiogenesis and aggressiveness of many disease types. Anti-VEGF strategies include the use of neutralizing antibodies to VEGF or its receptors, ribozymes targeted to receptor mRNA, and tyrosine kinase inhibitors that block downstream signaling. All the above-mentioned strategies have shown promise in preclinical trials and are now in clinical development.

One of the earliest strategies used to inhibit VEGF activity involved the use of a neutralizing antibody to VEGF where the antibody is a hybrid of a variable region that recognizes the epitope and a humanized Fc region that is not recognized as foreign by the human host. This latter region should also interact with human Fc-receptor-bearing effector cells and/or human complement. A similar strategy is utilized for anti-VEGF receptor antibodies. Antibodies must be delivered intravenously, although the long half-life may allow administration once every 2 or 3 weeks.

The other commonly used strategy for inhibiting VEGF activity is the use of tyrosine kinase inhibitors.[55] These are small molecules that prevent kinase activation on bind-

Note from the Editors—This is an update of Dr. Lee M. Ellis et al's original article which was published in *ONCOLOGY*, Volume 15, Number 7, Supplement No. 8, pages 39-46, July 2001

in the develop-
 enic therapies are
 distinct. Antian-
 can be classified
 tegories: (1) those
 ctivity of specific
 (2) those that de-
 of endothelial sur-
 pose that increase
 ally occurring an-
 such as angiosta-
 mbospondin; and
 rectly downregu-
 giogenic and sur-

v of
 rs
 discussion, VEGF
 pe molecule used
 es to decrease the
 ic factors because
 the angiogenesis
 of many disease
 strategies include
 zing antibodies to
 rs, ribozymes tar-
 RNA, and tyrosine
 hat block down-
 ll the above-men-
 ve shown promise
 s and are now in
 nt.

est strategies used
 ivity involved the
 zing antibody to
 itbody is a hybrid
 n that recognizes
 humanized Fc re-
 gnized as foreign
 This latter region
 t with human Fc-
 ffector cells and/
 ment. A similar
 for anti-VEGF re-

Antibodies must
 enously, although
 may allow admin-
 y 2 or 3 weeks.
 nonly used strate-
 VEGF activity is
 ne kinase inhibi-
 e small molecules
 activation on bind-

s—This is an update of
 iginal article which was
 Y, Volume 15, Number
 ges 39-46, July 2001.

ing of the ligand to a tyrosine kinase
 receptor. Although these compounds
 are claimed to be relatively selective
 for their specific targets, these ty-
 rosine kinase inhibitors actually do
 have some cross-reactivity with oth-
 er receptors, albeit requiring a much
 higher dose to achieve an effect.
 These inhibitors are delivered intra-
 venously or orally.

Anti-VEGF and Increased Apoptosis

Studies from our laboratory have
 examined anti-VEGF receptor anti-
 bodies and tyrosine kinase inhibitors
 in mouse models of colon cancer and
 liver metastasis.[54,56] Interest-
 ily, these agents demonstrated similar
 efficacy, leading to a decrease in he-
 patic tumor burden, vessel count, and
 proliferative index of the tumor cells.
 Surprisingly, we found an increase in
 the number of tumor cells undergo-
 ing apoptosis. We further investigat-
 ed this phenomenon to determine if
 endothelial cell apoptosis was the pre-
 mptive cause of tumor cell apopto-
 sis. We established a technique of
 double-staining whereby we could
 first identify endothelial cells and
 then identify those endothelial cells
 undergoing apoptosis using the ter-
 minal deoxynucleotidyl transferase-
 mediated deoxyuridine triphosphate
 nick-end labeling (TUNEL) assay.[54]

We found that a wave of endothe-
 lial cell apoptosis preceded a wave
 of tumor cells undergoing apopto-
 sis.[56] This suggests that endothe-
 lial cell apoptosis occurs prior to
 tumor cell apoptosis, demonstrating
 that VEGF is a survival factor for
 tumor endothelial cells and further
 supporting the hypothesis that main-
 tenance of the integrity of the tumor
 vasculature is required for tumor
 survival.

Because anti-VEGF therapy leads
 to an increase in tumor and endothe-

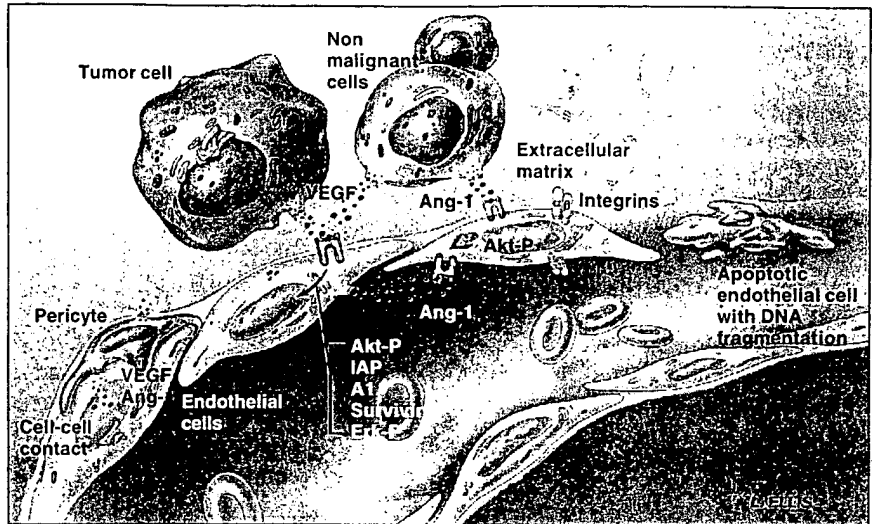


Figure 2: Endothelial Cell Survival Factors—Endothelial cell survival factors prevent apoptosis and include pericytes, vascular endothelial growth factor (VEGF), and angiopoietin-1 (Ang-1). Pericytes stabilize the endothelium by cell-to-cell contact or by the release of VEGF and Ang-1. VEGF inhibits endothelial cell apoptosis by activating a variety of intracellular signaling proteins including the Akt pathway, IAP, A1, and MAPK pathway. Reproduced with permission from Ellis et al.[58]

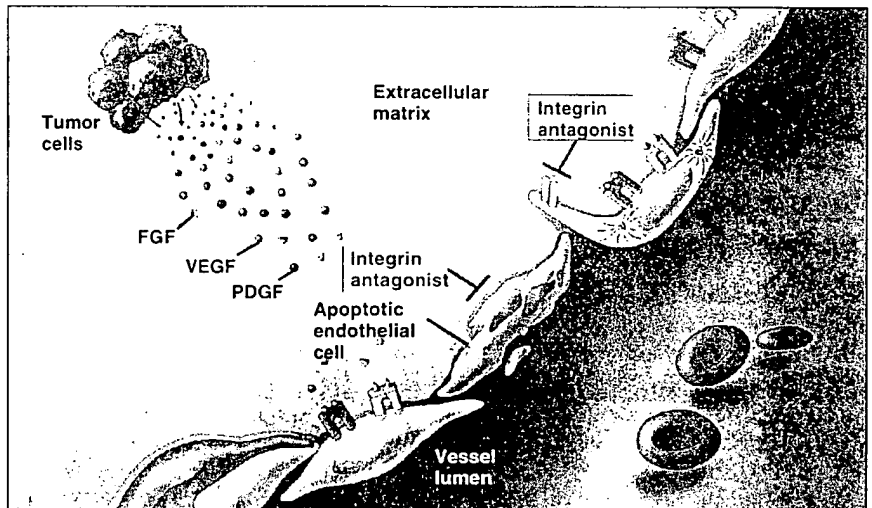


Figure 3: The Role of Integrin Antagonists in Preventing Attachment of Endothelial Cells to the Extracellular Matrix—Integrins, specifically alphav beta 3 and alphav beta 5, act as survival factors for endothelial cells. Disruption of the binding between the extracellular matrix and integrins leads to endothelial apoptosis. Small molecules and anti-integrin antibodies have been developed to block integrin/extracellular matrix interactions. FGF = fibroblast growth factor; VEGF = vascular endothelial cell growth factor; PDGF = platelet-derived growth factor. Used with permission from Pharmacia.

Address all correspondence to:
 LEE M. ELLIS, MD
 Departments of Surgical Oncology
 and Cancer Biology
 The University of Texas
 M.D. Anderson Cancer Center
 1515 Holcombe Blvd., Box 0444
 Houston, TX 77030-4009
 e-mail: lellis@mdanderson.org

lial cell apoptosis, one would sur-
 mise that this therapy could lead to a
 decrease in tumor size. There are re-
 ports of studies in subcutaneous xe-
 nograft models where tyrosine kinase
 inhibitors to the VEGF receptor and

other angiogenic factor receptors can
 cause regression of established tu-
 mors.[55] However, in our model of
 colon cancer liver metastasis, while
 tumor growth was inhibited, the
 growing cancer, albeit at a slower

rate, eventually led to the demise of the animals. This is likely due to the fact that there are redundant mechanisms for angiogenesis within tumors and that antiangiogenic therapy directed at a specific factor may lead to selection of cells whose angiogenesis is driven by a different factor.[3,57]

Decreased Activity of Endothelial Survival Factors

A second antiangiogenic strategy involves agents that decrease the activity of endothelial cell survival factors (Figure 2).[58] Typically, angiogenesis is simply thought of as the development of a new vasculature within tumors where endothelial cells migrate, proliferate, invade the basement membrane, and differentiate to form a primitive capillary network. However, the tumor microenvironment is a caustic one with low pH and low oxygen tension. Therefore, for these fragile endothelial cells to survive, they must be exposed to endothelial cell survival factors that prevent apoptosis in these adverse conditions.

Endothelial cell survival factors include pericytes that may stabilize endothelium, either by cell-to-cell contact or by secretion of endothelial cell survival factors such as VEGF or Ang-1. Vascular endothelial growth factor and Ang-1 are two endothelial cell survival factors that are necessary for endothelial cell survival in the absence of pericytes.[20] These factors can be secreted by endothelial cells, tumor cells, or nonmalignant cells within the microenvironment. Vascular endothelial growth factor has been shown to inhibit endothelial cell apoptosis by activation of various intracellular signaling proteins, including the Akt pathway, IAP, A1, and the MAPK pathway.[59] Angiopoietin-1 binds to the specific endothelial cell receptor, Tie-2, and activates the Akt pathway, a pathway that mediates survival in many cell types.[60]

Another very important mechanism for endothelial cell survival is the binding of integrins located on the endothelial cell surface to the extracellular matrix. At first, integrins were thought to be important only in cell-to-cell contact and bind-

ing to the extracellular matrix, but it is now known that integrins may mediate intracellular signaling, either alone or in combination with other receptors.[61] The integrins α v β 3, α v β 5, α v β 1 have been shown to act as survival factors for endothelial cells, and disruption of the binding between the integrins and the extracellular matrix may lead to endothelial cell death (Figure 3).[61-63] It is likely that integrin engagement with the extracellular matrix leads to integrin aggregation and activation of focal adhesion kinase. As a result, downstream signaling is activated, initiating endothelial cell survival mechanisms.[64]

Specific small molecules have been developed that may inhibit integrin activation, and antibodies have been synthesized that block integrin binding to the extracellular matrix.[65,66] Numerous agents are in preclinical evaluation or early clinical testing.

Increased Activity of Naturally Occurring Antiangiogenic Agents

Another antiangiogenic strategy is one that increases the activity of naturally occurring antiangiogenic agents. These agents include thrombospondin, angiostatin, and endostatin. A great deal of publicity has surrounded the discovery of angiostatin and endostatin, as these agents were first discovered as fragments of larger molecules (angiostatin is a fragment of plasminogen, and endostatin is a fragment of collagen XVI-II).[51,67,68] The exact mechanism by which these two compounds lead to a decrease in angiogenesis is not clearly understood. Thrombospondin, a naturally occurring angiogenic antagonist, is also being evaluated in preclinical trials.

The interferon family of proteins, although better known for other activities, also has antiangiogenic properties.[69-76] These cytokines, specifically interferon- α , were shown to cause regression of life-threatening childhood hemangiomas in a study published in the early 1990s.[72] Further investigation has demonstrated that interferon- α and interferon- β can downregulate basic fibroblast growth factor levels

in various tumor systems.[76] More recently, reports have demonstrated the efficacy of interferon- α in regression of tumors in children.[65] The efficacy of interferon therapy may be dependent on chronic low-dose therapy because higher dose therapy is often associated with intolerable side effects.

Indirect Downregulation of Angiogenic and Survival Factor Activity

The last antiangiogenic strategy is one that indirectly downregulates the activity of angiogenic factors. Vascular endothelial growth factor and other angiogenic factors are oftentimes unregulated in response to stress, such as hypoxia, low pH, or cytokines. Strategies that downregulate the upstream signaling pathways to VEGF and other angiogenic factors may indirectly downregulate VEGF activity and angiogenesis. Our laboratory, as well as others, has demonstrated that several growth factor receptors are involved in induction of VEGF on binding of its ligand to its receptor (EGF-R, IGF-receptor I).[36,77] Strategies to inhibit the activity of these receptors may lead to a decrease of in vivo VEGF production and angiogenesis, which in turn leads to a decrease in tumor growth.

It is also known that tumor suppressor genes, such as p53 and VHL, repress transcription of VEGF. We have shown that in a colon cancer cell line with a mutated p53 gene, infection of a wild-type p53 gene can lead to downregulation of VEGF and decrease angiogenesis in vivo.[38] It is possible that anti-VEGF therapy may be beneficial in patients with the VHL syndrome, which is almost certainly due to overexpression of VEGF in the formation of multiple vascular tumors.[43]

Future Directions in Antiangiogenic Therapy

Most local tumors can be adequately treated by surgery and/or radiation. However, the true challenge in oncology lies in treating metastatic cancers. The host microenvironment plays a major role in modulating

tems.[76] More
e demonstrated
ron-alpha in re-
n children.[65]
erferon therapy
n chronic low-
se higher dose
ociated with in-

ation survival

ogenic strategy
downregulates
ogenic factors,
growth factor
factors are of-
in response to
ia, low pH, or
that downregu-
lating pathways
angiogenic fac-
downregulate
giogenesis. Our
others. has dem-
l growth factor
ed in induction
of its ligand to
IGF-receptor
o inhibit the ac-
ors may lead to
VEGF produc-
s, which in turn
tumor growth.
hat tumor sup-
p53 and VHL,
of VEGF. We
a colon cancer
ated p53 gene,
pe p53 gene can
on of VEGF and
s in vivo.[38] It
-VEGF therapy
n patients with
which is almost
reexpression of
ion of multiple

in erapy

rs can be ade-
rgery and/or ra-
e true challenge
reating metastat-
t microenviron-
le in modulating

gene expression in tumors growing at different sites, and this holds true for angiogenic factor expression as well. In our laboratory, we have found that VEGF expression is actually higher in primary tumors than it is in liver metastases. Therefore, it would be naive for oncologists to think that antiangiogenic activity would be equally efficacious in different tumors growing at different sites. In addition, the endothelium is phenotypically distinct at different sites and, therefore, each tumor may not only express different angiogenic factors, but the endothelium may have different angiogenic factor receptors.[78]

Selection of Appropriate Therapy

It is foreseeable that in the future we will need to obtain biopsies of tumors growing at various metastatic sites and analyze expression of various genes within these biopsies. The revolution of microarray technology may allow us to rapidly identify angiogenic factors that may be driving angiogenesis in specific tumors at specific sites. At that point, we may then be able to direct appropriate antiangiogenic therapy toward specific targets. It is also possible that continued antiangiogenic therapy against a specific target may lead to selection of clones whose angiogenesis is driven by a different tumor. Therefore, it may be important to "re-stage" patients with repeat biopsy of these tumors to adequately determine the angiogenic profile of tumors within the course of their growth and, hopefully, response to antiangiogenic regimens. Clearly, inhibition of angiogenesis will play a substantial role in the future of oncology.

Supported in part by National Institutes of Health training grant T32 09599-08 (SA), the Gillson Longenbaugh Foundation (LME), the Jon and Suzie Hall Fund for Colon Cancer Research (LME), National Institutes of Health grant CA74821 (LME), the RGK Foundation (LME), the University Cancer Foundation (LME), and the National Institutes of Health Core Grant CCSG CA16672.

References

1. Weidner N: Tumor angiogenesis: Review of current applications in tumor prognostica-

tion. *New Cancer Strategies: Angiogenesis Antagonists* Washington, DC, April 3-4, 1995. Cambridge Healthtech Institute.

2. Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82:4-6, 1990.

3. Fidler IJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185-188, 1994.

4. Fidler IJ, Kerbel RS, Ellis LM: Biology of cancer: Angiogenesis; in DeVita VT Jr, Hellman S, Rosenberg SA (eds): *Cancer: Principles & Practice of Oncology*, 6th ed, vol 1, pp 137-147. Philadelphia, Lippincott Williams & Wilkins, 2001.

5. Takahashi Y, Kitadai Y, Bucana CD, et al: Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 55:3964-3968, 1995.

6. Takahashi A, Sasaki H, Kim SJ, et al: Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 54:4233-4237, 1994.

7. Takahashi Y, Cleary KR, Mai M, et al: Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. *Clin Cancer Res* 2:1679-1684, 1996.

8. Brown LF, Berse B, Jackman RW, et al: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 53:4727-4735, 1993.

9. Brown LF, Berse B, Jackman RW, et al: Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am J Pathol* 143:1255-1262, 1993.

10. Toi M, Kondo S, Suzuki H, et al: Quantitative analysis of vascular endothelial growth factor in primary breast cancer. *Cancer* 77:1101-1106, 1996.

11. Tischer E, Mitchell R, Hartman T, et al: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266:11947-11954, 1991.

12. Dvorak HF, Dvorak AM, Manseau EJ, et al: Fibrin gel investment associated with line 1 and line 10 solid tumor growth, angiogenesis, and fibroplasia in guinea pigs. Role of cellular immunity, myofibroblasts, microvascular damage, and infarction in line 1 tumor regression. *J Natl Cancer Inst* 62:1459-1472, 1979.

13. Senger DR, Galli SJ, Dvorak AM, et al: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219:983-985, 1983.

14. Dvorak HF, Nagy JA, Berse B, et al: Vascular permeability factor, fibrin, and the pathogenesis of tumor stroma formation. *Ann NY Acad Sci* 667:101-111, 1992.

15. Ziegler BL, Valtieri M, Porada GA, et al: KDR receptor: A key marker defining hematopoietic stem cells. *Science* 285:1553-1558, 1999.

16. Ferrer FA, Miller LJ, Lindquist R, et al: Expression of vascular endothelial growth factor receptors in human prostate cancer. *Urolo-*

gy 54:567-572, 1999.

17. Papapetropoulos A, Garcia-Cardena G, Dengler TJ, et al: Direct actions of angiopoietin-1 on human endothelium: Evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest* 79:213-223, 1999.

18. Lauren J, Gunji Y, Alitalo K: Is angiopoietin-2 necessary for the initiation of tumor angiogenesis? *Am J Pathol* 153:1333-1339, 1998.

19. Davis S, Yancopoulos GD: The angiopoietins: Yin and Yang in angiogenesis. *Curr Top Microbiol Immunol* 237:173-185, 1999.

20. Holash J, Maisonpierre PC, Compton D, et al: Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* 284:1994-1998, 1999.

21. Ahmad SA, Liu W, Jung YD, et al: The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. *Cancer Res* 61:1255-1259, 2001.

22. Etou T, Inoue H, Tanaka S, et al: Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: Possible in vivo regulation via induction of proteases. *Cancer Res* 61:2145-2153, 2001.

23. Hayes AJ, Huang WQ, Yu J, et al: Expression and function of angiopoietin-1 in breast cancer. *Br J Cancer* 83:1154-1160, 2000.

24. Moore BB, Arenberg DA, Addison CL, et al: Tumor angiogenesis is regulated by CXC chemokines. *J Lab Clin Med* 132:97-103, 1998.

25. Levy AP, Levy NS, Wegner S, et al: Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270:13333-13340, 1995.

26. Shweiki D, Neeman M, Itin A, et al: Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: Implications for tumor angiogenesis. *Proc Natl Acad Sci U S A* 92:768-772, 1995.

27. Shweiki D, Itin A, Soffer D, et al: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-845, 1992.

28. Ellis LM, Staley CA, Liu W, et al: Down-regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-Src. *J Biol Chem* 273:1052-1057, 1998.

29. Mukhopadhyay D, Tsiokas L, Zhou XM, et al: Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 375:577-581, 1995.

30. Laughner E, Taghavi P, Chiles K, et al: HER2/neu signaling increases the rate of hypoxia-inducible factor 1 alpha (HIF-1 alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* 21:3995-4004, 2001.

31. Williams CS, Mann M, DuBois RN: The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 18:7908-7916, 1999.

32. Tsujii M, Kawano S, Tsuji S, et al: Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 93:705-716, 1998.

33. Masferrer JL, Leahy KM, Koki AT, et al: Antiangiogenic and antitumor activities of cy-

clooxygenase-2 inhibitors. *Cancer Res* 60:1306-1311, 2000.

34. Bruns CJ, Solorzano CC, Harbison MT, et al: Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 60:2926-2935, 2000.

35. Ciardiello F, Caputo R, Bianco R, et al: Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 7:1459-1465, 2001.

36. Perrotte P, Matsumoto T, Inoue K, et al: Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 5:257-265, 1999.

37. Bruns CJ, Harbison MT, Davis DW, et al: Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin Cancer Res* 6:1936-1948, 2000.

38. Bouvet M, Ellis LM, Nishizaki M, et al: Adenovirus-mediated wild-type p53 gene transfer downregulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. *Cancer Res* 58:2288-2292, 1998.

39. Mazure NM, Chen EY, Laderoute KR, et al: Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* 90:3322-3331, 1997.

40. Maxwell PH, Dachs GU, Gleadle JM, et al: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci U S A* 94:8104-8109, 1997.

41. Jung YD, Nakano K, Liu W, et al: Extracellular signal-regulated kinase activation is required for upregulation of vascular endothelial growth factor by serum starvation in human colon carcinoma cells. *Cancer Res* 59:4804-4807, 1999.

42. Rak J, Mitsuhashi Y, Bayko L, et al: Mutant ras oncogenes upregulate VEGF/VPF expression: Implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 55:4575-4580, 1995.

43. Pal S, Claffey KP, Dvorak HF, et al: The von Hippel-Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. *J Biol Chem* 272:27509-27512, 1997.

44. Mukhopadhyay D, Knebelmann B, Cohen HT, et al: The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. *Mol Cell Biol* 17:5629-5639, 1997.

45. Levy AP, Levy NS, Goldberg MA: Hypoxia-inducible protein binding to vascular endothelial growth factor mRNA and its modulation by the von Hippel-Lindau protein. *J Biol Chem* 271:25492-25497, 1996.

46. Takahashi Y, Bucana CD, Cleary KR, et al: p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. *Int J Cancer* 79:34-38, 1998.

47. Abramovitch R, Dafni H, Neeman M, et al: Inhibition of neovascularization and tumor growth, and facilitation of wound repair, by halofuginone, an inhibitor of collagen type I synthesis. *Neoplasia* 1:321-329, 1999.

48. Klein SA, Bond SJ, Gupta SC, et al: Angiogenesis inhibitor TNP-470 inhibits murine cutaneous wound healing. *J Surg Res* 82:268-274, 1999.

49. Berger AC, Feldman AL, Gnani MF, et al: The angiogenesis inhibitor, endostatin, does not affect murine cutaneous wound healing. *J Surg Res* 91:26-31, 2000.

50. Bloch W, Hügge K, Sasaki T, et al: The angiogenesis inhibitor endostatin impairs blood vessel maturation during wound healing. *FASEB J* 14:2373-2376, 2000.

51. O'Reilly MS, Boehm T, Shing Y, et al: Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88:277-285, 1997.

52. Lode HN, Moehler T, Xiang R, et al: Synergy between an antiangiogenic integrin alphav antagonist and an antibody-cytokine fusion protein eradicates spontaneous tumor metastases. *Proc Natl Acad Sci U S A* 96:1591-1596, 1999.

53. Warren RS, Yuan H, Matli MR, et al: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95:1789-1797, 1995.

54. Shaheen RM, Davis DW, Liu W, et al: Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res* 59:5412-5416, 1999.

55. Laird AD, Vajkoczy P, Shawver LK, et al: SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res* 60:4152-4160, 2000.

56. Bruns CJ, Liu W, Davis DW, et al: Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. *Cancer* 89:488-499, 2000.

57. Takahashi Y, Bucana CD, Liu W, et al: Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: Role of infiltrating cells. *J Natl Cancer Inst* 88:1146-1151, 1996.

58. Ellis LM, Liu W, Ahmad SA, et al: Overview of angiogenesis: Biologic implications for antiangiogenic therapy. *Semin Oncol* 28(suppl 16):94-104, 2001.

59. Liu W, Ahmad SA, Reinmuth N, et al: Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis* 5:323-328, 2000.

60. Papadopoulos A, Fulton D, Mahboubi K, et al: Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* 275:9102-9105, 2000.

61. Eliceiri BP, Cheresh DA: The role of alphav integrins during angiogenesis: Insights into potential mechanisms of action and clinical development. *J Clin Invest* 103:1227-1230, 1999.

62. Eliceiri BP, Klemke R, Stromblad S, et al: Integrin alphav beta 3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *J Cell Biol* 140:1255-1263, 1998.

63. Scatena M, Almeida M, Chaisson ML, et al: NF-kappa B mediates alphav beta 3 integrin-induced endothelial cell survival. *J Cell Biol* 141:1083-1093, 1998.

64. Akiyama SK: Integrins in cell adhesion and signaling. *Hum Cell* 9:181-186, 1996.

65. Kaban LB, Mulliken JB, Ezekowitz RA, et al: Antiangiogenic therapy of a recurrent giant cell tumor of the mandible with interferon alfa-2a. *Pediatrics* 103:1145-1149, 1999.

66. Allman R, Cowburn P, Mason M: In vitro and in vivo effects of a cyclic peptide with affinity for the alpha(nu) beta 3 integrin in human melanoma cells. *Eur J Cancer* 36:410-422, 2000.

67. O'Reilly MS, Holmgren L, Shing Y, et al: Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79:315-328, 1994.

68. O'Reilly MS, Pirie-Shepherd S, Lane WS, et al: Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. *Science* 285:1926-1928, 1999.

69. Bielenberg DR, McCarty MF, Bucana CD, et al: Expression of interferon-beta is associated with growth arrest of murine and human epidermal cells. *J Invest Dermatol* 112:802-829, 1999.

70. Dinney CP, Bielenberg DR, Perrotte P, et al: Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 58:808-814, 1998.

71. Ellis LM, Fidler IJ: Angiogenesis and metastasis. *Eur J Cancer* 32A:2451-2460, 1996.

72. Ezekowitz RA, Mulliken JB, Folkman J: Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med* 326:1456-1463, 1992.

73. Kerbel RS, Vitoria-Petit A, Klement G, et al: 'Accidental' antiangiogenic drugs. Anticancer directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. *Eur J Cancer* 36:1248-1257, 2000.

74. Ohlms LA, Jones DT, McGill TJ, et al: Interferon alfa-2a therapy for airway hemangiomas. *Ann Otol Rhinol Laryngol* 103:1-8, 1994.

75. Ruegg C, Yilmaz A, Bieler G, et al: Evidence for the involvement of endothelial cell integrin alphav beta 3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med* 4:408-414, 1998.

76. Singh RK, Gutman M, Bucana CD, et al: Interferons alpha and beta downregulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci U S A* 92:4562-4566, 1995.

77. Akagi Y, Liu W, Zebrowski B, et al: Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I. *Cancer Res* 58:4008-4014, 1998.

78. Pasqualini R, Ruoslahti E: Organ targeting in vivo using phage display peptide libraries. *Nature* 380:364-366, 1996.

FRANCIS
Chief,
Develop
Associat
Depart
The Un
M. D.
Housto

D
acute
chronic
acute
chronic
the m
(MDS,
(NHL)
myelo
metapl
nential
marrow
is sign
advanc
mia w
RAES
comp
Pat
greater
success
has re
in bor
tients.
compl
with a
MVD
sociat
MVD
and M
increa
the n
nance
cove
arbori
with
MVD
with
dex.[]

The role of vascular endothelial growth factor in pathological angiogenesis

Napoleone Ferrara

Department of Cardiovascular Research, Genentech, Inc., South San Francisco CA, USA

Key words: vascular endothelial growth factor, endothelium, angiogenesis, tumor growth, ischemic disorders, retinopathy, rheumatoid arthritis

Summary

Vascular endothelial growth factor (VEGF) is a diffusible endothelial cell-specific mitogen and angiogenic factor that can also increase vascular permeability. By alternative splicing of mRNA, VEGF may exist as one of four different isoforms that have similar biological activities but differ markedly in targeting and bioavailability. The VEGF receptors are specifically expressed in the cell surface of vascular endothelial cells. Recent studies point to VEGF as a major regulator of physiological angiogenesis, such as developmental and reproductive angiogenesis. Furthermore, VEGF appears to be a crucial mediator of blood vessel growth associated with tumors and proliferative retinopathies. The VEGF mRNA is up-regulated in the majority of human tumors and the VEGF protein is increased in the aqueous and vitreous humors of patients with proliferative retinopathies. Anti-VEGF antibodies have the ability to suppress the growth of a variety of tumor cell lines in nude mice and also can inhibit angiogenesis in animal models of intraocular neovascularization. Therefore, strategies aimed at antagonizing VEGF may form the basis for an effective treatment of tumors and retinopathies. Furthermore, VEGF-induced angiogenesis is sufficient to achieve a therapeutic endpoint in models of coronary or limb ischemia.

Introduction

Angiogenesis is prominent during embryonic development and somatic growth, but in a normal adult it only takes place following injury or, in a cyclical fashion, in the endometrium and in the ovary [1-3]. Angiogenesis plays an important role in the pathogenesis of a variety of disorders such as cancer, proliferative retinopathies, and rheumatoid arthritis [1-3]. In the case of neoplasms, the new blood vessels provide nourishment to the growing tumor and also allow the tumor cells to establish continuity with the

vasculature of the host [1-3]. A strong correlation exists between density of microvessels in primary breast carcinoma sections and nodal metastases and survival [4-7]. Also, a correlation has been noted between vascularity and aggressive behavior in bladder [8], prostate [9,10], non-small cell lung [11], and uterine cervix [12] carcinomas, and in cutaneous melanomas [13]. Furthermore, recent studies have shown a statistically significant increase in microvessel count in severe uterine cervix dysplasia (CIN III) as compared with low grade lesions (CIN I) [14]. Therefore, the number of vessels in tumor sections, which reflects the

Address for correspondence and offprints: Napoleone Ferrara, Department of Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA; *Tel:* 415-225-2968; *Fax:* 415-225-6327

extent of angiogenesis, may be considered as an important independent predictor of outcome in breast cancer and, possibly, in other malignancies.

Angiogenesis requires at least three steps: a) degradation of the extracellular matrix of a venule; b) chemotaxis of endothelial cells toward an angiogenic stimulus; and c) proliferation of endothelial cells [1-3]. A variety of factors have been previously identified as potential positive regulators of angiogenesis: aFGF, bFGF, EGF, TGF α , TGF β , PGE $_2$, monobutyrin, HGF, TNF α , PD-ECGF, angiogenin, and interleukin-8 [1-3]. Some of these factors are able to directly stimulate endothelial cell growth, while others lack direct stimulatory effects on endothelial cells and thus their angiogenic actions are thought to require the paracrine release of direct-acting factors from macrophages or other cells [1-3].

This article will review the molecular properties of vascular endothelial growth factor (VEGF), a recently identified directly-acting endothelial cell mitogen and angiogenesis inducer [15-18], and will discuss the role of this factor in the regulation of blood vessel growth. By alternative splicing of mRNA, multiple molecular forms of VEGF are generated [19-22]. Several lines of evidence indicate that VEGF is a major regulator of physiological angiogenesis, such as embryonic [23,24] and reproductive [25-27] angiogenesis. Also, VEGF-induced angiogenesis is sufficient to achieve a therapeutic endpoint in animal models of coronary [28] or limb [29,30] ischemia. Furthermore, recent studies point to VEGF as a crucial mediator of neovascularization associated with tumors and proliferative retinopathies [31-34].

Biological activities of VEGF

VEGF is a potent mitogen (ED $_{50}$ 2-10 pM) for vascular endothelial cells, but it is apparently devoid of appreciable mitogenic activity for other cell types [15,16,18]. Consequently, VEGF has been regarded as an endothelial cell-specific mitogen. VEGF is also able to induce a marked

angiogenic response in the chick chorioallantoic membrane [16,18]. VEGF also promotes angiogenesis in a tridimensional *in vitro* model, inducing confluent microvascular endothelial cells to invade a collagen gel and form tube-like structures [35]. These studies also provide evidence for a potent synergism between VEGF and bFGF in the induction of such *in vitro* angiogenic effects [35]. Recently, it has been proposed that VEGF may function as a paracrine mediator for an indirect-acting angiogenic agent like TGF β [36]. Treatment of quiescent cultures of epithelial and fibroblastic cells with TGF β results in induction of VEGF mRNA and release of VEGF protein into the medium [36]. VEGF has the ability to promote vascular permeability, as evidenced by Evans blue extravasation, when injected in the guinea pig skin [17,20]. Based on this activity, the factor has been proposed to be a specific mediator of the hyperpermeability of tumor vessels. It has been suggested that extravasation of fibrinogen and other plasma proteins results in the formation of a fibrin gel that serves as a substrate for endothelial and tumor growth [37]. Additionally, VEGF induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor-1 (PAI-1) in cultured bovine microvascular endothelial cells [38]. Furthermore, VEGF induces expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts [39]. Interstitial collagenase is able to degrade type I and III collagen [40]. The co-induction of plasminogen activators and collagenase by VEGF is expected to promote a pro-degradative environment that facilitates migration of endothelial cells. The expression of PAI-1 may serve to regulate and balance the process [41]. VEGF has also been shown to induce vasodilatation in a dose-dependent fashion [42], which translates into a transient hypotension *in vivo*. Such an effect appears to be mediated by endothelial cell-derived NO, as assessed by the requirement for an intact endothelium and the prevention of the effect by N-methyl-arginine [42].

Recent studies [43] indicate that the *in vivo* and *in vitro* bioactivity of VEGF can be potentiated by placenta growth factor (PlGF), a molecule having a significant degree of structural homology with VEGF [44,45]. While PlGF has little or no direct mitogenic or permeability-enhancing activity, it is able to significantly potentiate the activity of low, marginally efficacious concentrations of VEGF [43]. This effect requires a 10-20 fold molar excess of PlGF over VEGF. Therefore, it may be that PlGF serves to enhance the bioactivity of VEGF in situations where the concentrations of the latter are limiting [43].

Structural and genomic properties of VEGF

VEGF purified from a variety of species and sources is a basic, heparin-binding, homodimeric glycoprotein of 45,000 daltons [15,16,18]. VEGF is inactivated by reducing agents, but it is heat-stable and acid-stable. cDNA sequence analysis of a variety of VEGF clones indicates that VEGF may exist as one of four different molecular species, having respectively 121, 165, 189, and 206 amino acids (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆) [19-22]. VEGF₁₆₅ is the predominant isoform secreted by a variety of normal and transformed cells. The biochemical properties of recombinant VEGF₁₆₅ correspond closely to those of native VEGF purified from a variety of sources [16]. In addition, transcripts encoding VEGF₁₂₁ and VEGF₁₈₉ are detected in the majority of cells and tissues expressing the VEGF gene [21]. By contrast, VEGF₂₀₆ is a very rare form, so far identified only in a human fetal liver cDNA library [21]. Compared to VEGF₁₆₅, VEGF₁₂₁ lacks 44 amino acids, VEGF₁₈₉ has an insertion of 24 amino acids highly enriched in basic residues, and VEGF₂₀₆ has an additional insertion of 17 amino acids. It is now known that alternative splicing of RNA, rather than transcription of separate genes, is the basis for the molecular heterogeneity evidenced by the cDNA sequence analysis [21,22]. The VEGF gene is organized in

eight exons and the size of its coding region has been estimated to be approximately 14 kb [22]. VEGF₁₆₅ lacks the residues encoded by exon 6, while VEGF₁₂₁ lacks the residues encoded by exons 6 and 7.

Hypoxia has been recently identified as an important mechanism involved in the regulation of the expression of the VEGF gene, both *in vivo* and *in vitro* [46,47]. Recent studies have shown that similarities exist between the oxygen-sensing mechanisms regulating the expression of VEGF and erythropoietin genes [47]. The expression of both genes is significantly enhanced by cobalt chloride. Furthermore, the hypoxic induction of both VEGF and erythropoietin genes is inhibited by carbon monoxide, suggesting the involvement of a heme protein in the process of sensing oxygen levels [47].

The VEGF isoforms

VEGF₁₂₁ is a weakly acidic polypeptide that fails to bind to heparin [48]. In contrast, VEGF₁₆₅ is a basic, heparin-binding protein [48]. VEGF₁₈₉ and VEGF₂₀₆ are still more basic and bind to heparin with even greater affinity [48]. Such differences in the isoelectric point and in affinity for heparin profoundly affect the bioavailability of the VEGF isoforms [48,49]. VEGF₁₂₁ is secreted as a freely soluble protein in the conditioned medium of transfected cells. VEGF₁₆₅ is also secreted, but a significant fraction remains bound to the cell surface or the extracellular matrix (ECM). In contrast, VEGF₁₈₉ and VEGF₂₀₆ are almost completely sequestered in the ECM [49]. However, they may be released from the bound state by a variety of agents such as suramin, heparin, or heparinase [48,49]. ECM prepared from cells expressing VEGF₁₈₉ or VEGF₂₀₆ promotes the growth of vascular endothelial cells [49]. The observation that heparin releases these forms of VEGF suggests that their binding site is represented by heparin-containing proteoglycans, similar to the binding site for bFGF. Furthermore, the long forms may be released by plasmin

[48,49]. This physiologically relevant protease is able to cleave VEGF₁₈₉ or VEGF₂₀₆ at the COOH terminus and generate a proteolytic fragment having molecular weight of ~34,000 daltons which is active as an endothelial cell mitogen and as a vascular permeability agent [48]. Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade [50]. It is possible that this property is not confined to plasmin. It may be that cleavage of VEGF may be brought about by a variety of inflammation-associated proteases. Thus, the VEGF proteins may become available to endothelial cells by at least two different mechanisms: as freely diffusible proteins (VEGF₁₂₁, VEGF₁₆₅) or following protease activation and cleavage of the longer isoforms. Generation of bioactive VEGF by proteolytic cleavage may be especially important in the microenvironment of a tumor, where increased expression of proteases, including plasminogen activators, is well documented [51,52]. Therefore, the products of a single gene may provide a highly flexible system to control angiogenesis. The short forms of VEGF may be rapidly and efficiently released in response to the requirements of the microenvironment. In contrast, the longer forms are stably incorporated in the ECM but can become available in a diffusible form when this structure is degraded.

The VEGF receptors

Characteristics and distribution of VEGF binding sites

Two classes of high affinity VEGF binding sites have been identified on the cell surface of cultured endothelial cells, having K_d values of 10 pM and 100 pM, respectively [53,54]. Lower affinity binding sites on mononuclear phagocytes (K_d ~300-500 pM) have been recently described [55]. It has been suggested that such binding sites are involved in mediating chemotactic effects for monocytes by VEGF [55].

Ligand autoradiography studies on tissue sections of fetal [24] or adult [56] rats have shown that high affinity ¹²⁵I-VEGF binding sites are localized to the vascular endothelium of large or small vessels but not to other cell types. Scatchard analysis of saturation isotherms in sections from a variety of tissues revealed a single class of binding sites with high affinity (K_d = 16 to 35 pM) [56]. Specific binding co-localized with Factor VIII-like immunoreactivity and was apparent on both proliferating and quiescent endothelial cells [56]. Binding of ¹²⁵I-VEGF during development of rat embryos is first detectable in the blood islands of the yolk sac, which contain the earliest progenitors of hematopoietic and endothelial cells [24]. As the vascular system develops, VEGF binding sites continue to co-localize with the endothelium of blood vessels [24,56].

The Flt-1 and Flk-1/KDR tyrosine kinases

Two tyrosine kinases have been identified as putative VEGF receptors [57-60]. The Flt-1 (fms-like-tyrosine kinase) [61] and KDR (kinase domain region) [62] proteins have been shown to bind VEGF with high affinity. The overall amino acid sequence identity between the two proteins is 44%. The murine homologue of KDR is known as Flk-1 (fetal liver kinase-1) [63] and shares 85% sequence identity with human KDR. Both Flt-1 and KDR/Flk-1 have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane domain, and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain [61-63]. Flt-1 has the highest affinity for rhVEGF₁₆₅, with a K_d of approximately 10-20 pM [57]. KDR has a somewhat lower affinity for VEGF; the K_d has been estimated to be approximately 75-125 pM [43,58]. The K_d for binding of rhVEGF₁₆₅ to Flk-1 is 500-600 pM [59,60].

Recently, an alternatively spliced form of Flt-1, lacking the seventh immunoglobulin-like domain, the transmembrane sequence, and the

cytoplasmic domain, has been identified in human umbilical vein endothelial cells [64]. The encoded protein is soluble following secretion. This soluble Flt-1 receptor is able to inhibit VEGF-induced mitogenesis and has been proposed to be a physiological negative regulator of VEGF action [64].

In situ hybridization studies have revealed that the Flk-1 mRNA is expressed in the vascular endothelium in the mouse embryo [59,60]. There is evidence that the Flk-1 mRNA is down-regulated in adult endothelial cells as compared to fetal endothelial cells [60]. The Flt-1 mRNA is selectively expressed in vascular endothelial cells, both in fetal and adult mouse tissues [65]. Like the high affinity VEGF binding [24,56], the Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells [65].

Recent studies suggest that the Flt-1 and KDR proteins may have different signal transduction properties [66]. Porcine aortic endothelial cells lacking endogenous VEGF receptors display chemotaxis and mitogenesis in response to VEGF when transfected with an expression vector coding for KDR. In contrast, transfected cells expressing Flt-1 lack such responses [66]. While Flk-1/KDR undergoes ligand-dependent tyrosine phosphorylation in intact cells [59,60,66], Flt-1 fails to show such a response [57,65]. These findings are in agreement with other studies showing that PlGF, which binds with high affinity to Flt-1 but not to Flk-1/KDR, lacks direct mitogenic or permeability-enhancing properties or the ability to stimulate tyrosine phosphorylation in endothelial cells [43]. Therefore, it appears that interaction with Flk-1/KDR is a critical requirement to induce the full spectrum of VEGF biologic responses. Whether VEGF induces formation of heterodimers between Flt-1 and Flk-1/KDR, which could confer new properties or ligand specificities upon these receptors, remains to be established. Further studies are clearly required to characterize the molecular events involved in VEGF signal transduction.

Role of VEGF in tumor angiogenesis

Numerous tumor cell lines express the VEGF mRNA and secrete a VEGF-like protein into the medium [67,68]. Furthermore, *in situ* hybridization studies have shown that the VEGF mRNA is markedly up-regulated in a variety of human tumors, including kidney [69], ovarian [70], and gastrointestinal [71] carcinomas, and in several intracranial tumors including glioblastoma multiforme [46,72,73] and capillary hemangioblastoma [74]. In all of these circumstances, the VEGF mRNA is expressed in tumor cells but not in endothelial cells, indicating that VEGF is a purely paracrine mediator. However, immunohistochemical studies have localized the VEGF protein not only to the tumor cells but also to the vasculature [71,72]. This localization indicates that tumor-secreted VEGF accumulates in the target cells. A strong correlation exists between degree of vascularization of the malignancy and VEGF mRNA expression [46,72,74]. In addition, the mRNA for the VEGF receptors, Flt-1 and KDR, is up-regulated in the tumor-associated endothelial cells in comparison with the vasculature of the surrounding tumor-free tissue [71,72]. Recent studies indicate that the VEGF and PlGF mRNAs are co-expressed in renal cell carcinoma, suggesting that, at least in this tumor, the two factors may play a cooperative role in the induction of angiogenesis [69]. Interestingly, a correlation has been observed between VEGF expression, as assessed by immunohistochemistry, and microvessel density in primary breast cancer sections [75]. Postoperative survey indicated that the relapse-free survival rate of VEGF-rich tumors was significantly worse than that of VEGF-poor tumors, suggesting that expression of VEGF is associated with stimulation of angiogenesis and with early relapse in primary breast cancer [75]. In tumors with a significant component of necrosis, such as glioblastoma multiforme, VEGF mRNA expression is not uniform but occurs primarily in clusters of tumor cells at the border between viable tumor and necrotic areas [46,72,73]. This localization is consistent with local

hypoxia being a major inducer of VEGF gene expression [46,47], and suggests that a VEGF gradient is responsible for angiogenesis and tumor expansion toward ischemic areas.

Taken together, these findings suggest that the VEGF-expressing tumor cells may have a growth advantage *in vivo* due to stimulation of angiogenesis. This hypothesis is consistent with previous studies showing that expression of VEGF₁₆₅ or VEGF₁₂₁ confers on a non-tumorigenic clone of Chinese hamster ovary cells the ability to form vascularized tumors in nude mice [76]. However, VEGF expression did not result in a growth advantage *in vitro* for such cells, indicating that their ability to grow *in vivo* was due to paracrine rather than autocrine mechanisms. More direct evidence for a role of VEGF in tumor angiogenesis has recently been made possible by the availability of specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis *in vivo* and *in vitro* [31]. Such antibodies exert a dramatic inhibitory effect on the growth of a variety of human tumor cell lines injected subcutaneously in nude mice, including glioblastoma multiforme, rhabdomyosarcoma, and leiomyosarcoma [31]. However, the antibodies (or VEGF) have no effect on the *in vitro* growth of the tumor cells. The density of vessels, as assessed by immunostaining for Factor VIII-like antigen, was significantly decreased in tumor sections of antibody-treated animals as compared with controls [31]. The concept that inhibition of VEGF action results in suppression of tumor growth has been confirmed by the finding that a negative dominant Flk-1 mutant inhibits glioblastoma growth *in vivo* [77]. More recently, it has been shown that VEGF is a major mediator of the *in vivo* growth of human colon carcinoma cells in a nude mouse model of liver metastasis where the tumor cells are injected in the spleen [78]. As in human tumors, the expression of Flk-1 mRNA in this murine model was markedly up-regulated in the vasculature associated with liver metastases. Treatment with anti-VEGF monoclonal antibodies resulted in a dramatic decrease in the number and size of metastases.

Most of the tumors in the treated group were under 1 mm in diameter and all were under 3 mm. Also, neither blood vessels nor Flk-1 mRNA expression could be demonstrated in such metastases. These findings are in good agreement with the hypothesis that growth of a tumor beyond 2-3 mm requires neovascularization [1-3]. Administration of anti-VEGF antibodies also results in dramatic suppression of gross peritoneal tumor and ascites following injection of ovarian carcinoma cells in the peritoneal cavity of nude mice [79]. These findings indicate that VEGF-induced angiogenesis is a critical rate-limiting step even in the process of ovarian cancer tumorigenesis.

Role of VEGF in angiogenesis associated with other disorders

Intraocular neovascularization associated with diabetes, occlusion of the central retinal vein, or prematurity and exposure to oxygen can lead to vitreous hemorrhage, retinal detachment followed by eventual blindness, and neovascular glaucoma [80]. Diabetic retinopathy is the leading cause of blindness in the western world [80]. All of these conditions are known to be associated with retinal ischemia [80]. As early as 1948, Michaelson proposed that the ischemic retina is able to release into the vitreous diffusible angiogenic factor(s) responsible for retinal and iris neovascularization [81]. Even though IGF-1 and bFGF have been implicated in this process, these factors do not show a consistent increase as would be expected if they played a significant causative role [82,83]. On the other hand, VEGF, by virtue of its diffusible nature and hypoxia-inducibility, is an attractive candidate as a retina-derived mediator of intraocular neovascularization. Recently, elevations of VEGF levels in the aqueous and vitreous of eyes with proliferative retinopathy have been reported [33,84]. In a large series where 164 patients and 210 samples of ocular fluid were examined, a strong correlation was found between levels of immunoreactive VEGF in the aqueous

and vitreous humors and active proliferative retinopathy [33]. VEGF levels were undetectable or very low (<0.5 ng/ml) in the eyes of individuals affected by non-neovascular disorders or diabetes without proliferative retinopathy [33]. In contrast, the VEGF levels were in the range of 3-10 ng/ml in the presence of active proliferative retinopathy associated with diabetes, occlusion of central retinal vein, or prematurity. Remarkably, the VEGF levels were very low in the eyes of patients with quiescent proliferative retinopathy, suggesting a temporal correlation between VEGF release in the vitreous and blood vessel growth. These findings are consistent with the hypothesis that ischemia in the retina, regardless of its etiology, leads to release of VEGF, followed by neovascularization. More direct evidence for this hypothesis has been provided in a primate model of iris neovascularization that closely mimics human disease [85]. In this model, intraocular administration of anti-VEGF antibodies is able to dramatically inhibit the neovascularization that follows occlusion of central retinal veins [34]. These findings suggest that treatment with inhibitors of VEGF action may prevent the consequences of neovascularization associated with diabetes or other ischemic retinal disorders.

It has been also proposed that VEGF is involved in the pathogenesis of another important disease where angiogenesis plays a significant role, rheumatoid arthritis (RA) [86,87]. The RA synovium is characterized by the formation of pannus, an extensively vascularized tissue that invades and destroys the articular cartilage [88]. By its vascularity and rapid proliferation rate, the RA synovium has been likened to a tumor [89]. Levels of immunoreactive VEGF are high in the synovial fluid of RA patients while they are very low or undetectable in the synovial fluid of patients affected by other forms of arthritis or by degenerative joint disease. Furthermore, anti-VEGF antibodies significantly reduce the endothelial cell chemotactic activity of the RA synovial fluid, indicating that immunoreactive VEGF is bioactive [86].

Conclusions

VEGF has been identified and characterized only recently but already appears to play a pivotal role in the regulation of blood vessel growth as well as having considerable therapeutic potential. An attractive possibility is that the VEGF protein or gene therapy with VEGF may be used to promote revascularization in conditions of insufficient tissue perfusion. For example, chronic limb ischemia, most frequently caused by obstructive atherosclerosis affecting the superficial femoral artery, is associated with a high rate of morbidity and mortality and treatment is currently limited to surgical revascularization or endovascular interventional therapy [90,91]. Recent studies already indicate that intra-arterial administration of rhVEGF₁₆₅ [29] or arterial gene transfer of VEGF [30] may significantly augment perfusion and development of collateral vessels in a rabbit model of hindlimb ischemia. Furthermore, VEGF administration results in increased coronary blood flow in a dog model of coronary insufficiency [28].

The high expression of VEGF mRNA in many human tumors [69-72], and the presence of the VEGF protein in ocular fluids of individuals with proliferative retinopathies [33] and in the synovial fluid of RA patients [86], strongly support the hypothesis that VEGF is a key mediator of angiogenesis associated with various pathological conditions. Therefore, anti-VEGF antibodies or VEGF antagonists have the potential to be of therapeutic value for a variety of highly vascularized and aggressive malignancies as well as for other angiogenic disorders. An anti-VEGF therapy is expected to have low toxicity, perhaps limited to inhibition of wound healing and ovarian and endometrial function, since endothelial cells are essentially quiescent in most adult tissues.

In conclusion, recent evidence strongly suggests that, in spite of the plurality of factors potentially involved in pathological angiogenesis [1-3], strategies aimed at antagonizing one specific endothelial cell mitogen, VEGF, may

form the basis for an effective treatment of a variety of tumors and proliferative retinopathies.

References

1. Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267:10931-10934, 1992.
2. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. *Annu Rev Physiol* 53:217-239, 1991.
3. Folkman J: What is the evidence that tumors are angiogenesis-dependent? *J Natl Cancer Inst* 82:4-6, 1991.
4. Weidner N, Semple P, Welch W, Folkman J: Tumor angiogenesis and metastasis. Correlation in invasive breast carcinoma. *New Engl J Med* 324:1-6, 1991.
5. Vartanian RK, Weidner N: Correlation of intramural endothelial cell proliferation with microvessel density (tumor angiogenesis) and tumor cell proliferation. *Am J Pathol* 144:1188-1194, 1994.
6. Horak ER, Leek R, Klenk N, Lejeune S, Smith K, Stuart M, Greenall M, Harris AL: Quantitative angiogenesis assessed by anti-PECAM antibodies: correlation with node metastasis and survival in breast cancer. *Lancet* 340:1120-1124, 1992.
7. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G: Tumor angiogenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 84:1875-1888, 1992.
8. Chodak GW, Haudenschild C, Gittes RF, Folkman J: Angiogenic activity as a marker of neoplastic and preneoplastic lesions of the human bladder. *Ann Surg* 192:762-771, 1980.
9. Wakui S, Furusato M, Sasaki H, Akiyama A, Kinoshito I, Asano K, Tokuda T, Aizawa S, Ushigome S: Tumor angiogenesis in prostatic carcinoma with and without bone metastasis: a morphometric study. *J Pathol* 168:257-262, 1992.
10. Bigler SA, Deering RE, Brawer MK: Comparison of microscopic vascularity in benign and malignant prostatic tissue. *Human Pathol* 24:220-226, 1993.
11. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA: Relation of neovascularization to metastasis of non-small cell lung carcinoma. *Lancet* 340:145-146, 1992.
12. Sillman F, Boyce J, Fruchter R: The significance of atypical vessels and neovascularization in cervical neoplasias. *Am J Obstet Gynecol* 139:154-157, 1981.
13. Srivastava A, Laidler P, Davies R, Horgan K, Hughes LE: The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma. *Am J Pathol* 133:419-423, 1988.
14. Smith-McCune KS, Weidner N: Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 54:804-808, 1994.
15. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851-859, 1989.
16. Ferrara N, Houck K, Jakeman L, Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13:18-32, 1992.
17. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 84:1470-1478, 1989.
18. Plouët J, Schilling J, Gospodarowicz D: Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT20 cells. *EMBO J* 8:3801-3807, 1989.
19. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306-1309, 1989.
20. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to platelet derived growth factor. *Science* 246:1309-1312, 1989.
21. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 5:1806-1814, 1991.
22. Tisher E, Mitchell R, Hartmann T, Silva M, Gospodarowicz D, Fiddes J, Abraham J: The human gene for vascular endothelial growth factor. *J Biol Chem* 266:11947-11954, 1991.
23. Breier G, Albrecht U, Sterrer S, Risau W: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114:521-532, 1992.
24. Jakeman LB, Armanini M, Phillips HS, Ferrara N: Developmental expression of binding sites and mRNA for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology* 133:848-859, 1993.
25. Phillips HS, Hains J, Leung DW, Ferrara N: Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 127:965-968, 1990.
26. Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznick AJ: Vascular endothelial growth factor mRNA expression in the primate ovary. *Endocrinology* 131:254-260, 1992.

27. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E: Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 91:2235-2243, 1993.
28. Banai S, Jaktlish MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein S, Unger E: Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* 89:2183-2189, 1994.
29. Takeshita S, Zhung L, Brogi E, Kearney M, Pu L-Q, Bunting S, Ferrara N, Symes JF, Isner JM: Therapeutic angiogenesis: A single intra-arterial bolus of vascular endothelial growth factor augments collateral vessel formation in a rabbit ischemic hindlimb model. *J Clin Invest* 93:662-670, 1994.
30. Takeshita S, Zheng LP, Cheng D, Riessen R, Weir L, Symes JF, Ferrara N, Isner JM: Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. *Proc Natl Acad Sci USA*, in press.
31. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362:841-844, 1993.
32. Ferrara N: Vascular endothelial growth factor. *Trends Cardiovasc Med* 3:244-250, 1993.
33. Aiello LP, Avery R, Arrigg R, Keyt B, Jampel H, Shah S, Pasquale L, Thieme H, Iwamoto M, Park JE, Nguyen H, Aiello LM, Ferrara N, King GL: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480-1487, 1994.
34. Adamis AP, Shima DT, Tolentino M, Gragoudas E, Ferrara N, Folkman J, D'Amore PA, Miller JW: Inhibition of VEGF prevents ocular neovascularization in a primate model. Submitted.
35. Pepper MS, Ferrara N, Orci L, Montesano R: Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochem Biophys Res Commun* 189:824-831, 1992.
36. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor- β in fibroblastic and epithelial cells. *J Biol Chem* 269:6271-6274, 1994.
37. Dvorak HF: Tumors: wounds that do not heal. Similarity between tumor stroma generation and wound healing. *N Engl J Med* 315:1650-1658, 1986.
38. Pepper MS, Ferrara N, Orci L, Montesano R: Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor type I in microvascular endothelial cells. *Biochem Biophys Res Commun* 181:902-908, 1991.
39. Unemori E, Ferrara N, Bauer EA, Amento EP: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 153:557-562, 1992.
40. Gross J, Nagai Y: Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc Natl Acad Sci USA* 54:1197-1204, 1965.
41. Pepper MS, Montesano R: Proteolytic balance and capillary morphogenesis. *Cell Diff Dev* 32:319-331, 1990.
42. Ku DD, Zaleski JK, Liu S, Brock T: Vascular endothelial growth factor induces EDRF-dependent relaxation of coronary arteries. *Am J Physiol* 265: H586-592, 1993.
43. Park JE, Chen H, Winer J, Houck K, Ferrara N: Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 269:25646-25654, 1994.
44. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG: Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA* 88: 9267-9271, 1991.
45. Hauser S, Weich HA: A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 9:259-268, 1993.
46. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-845, 1992.
47. Goldberg MA, Schneider TJ: Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 269:4355-4361, 1994.
48. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N: Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 267:26031-26037, 1992.
49. Park JE, Keller G-A, Ferrara N: The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of ECM-bound VEGF. *Mol Biol Cell* 4:1317-1326, 1993.
50. Mignatti P, Tsuboi R, Robbins E, Rifkin DB: In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. *J Cell Biol* 108:671-682, 1989.
51. Rifkin DB, Moscatelli D, Bizik J, Quarto N, Blei F, Dennis P, Flaumenhaft R, Mignatti P: Growth factor control of extracellular proteolysis. *Cell Differ Dev* 32:313-318, 1990.
52. Sreenath T, Matrisian LM, Stetler-Stevenson W,

- Gattoni-Celli S, Pozzatti RO: Expression of matrix metalloproteinases in transformed rat cell lines of high and low metastatic potential. *Cancer Res* 52:4942-4947, 1992.
53. Vaisman N, Gospodarowicz D, Neufeld G: Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem* 265:19461-19469, 1990.
 54. Ploüet J, Moukadi HJ: Characterization of the receptors to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. *J Biol Chem* 265:22071-22075, 1990.
 55. Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly DT, Stern D, Kao J: Characterization of vascular permeability factor/vascular endothelial growth factor receptors in mononuclear phagocytes. *Blood* 81:2767-2773, 1993.
 56. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest* 89:244-253, 1992.
 57. deVries C, Escobedo JA, Ueno H, Houck KA, Ferrara N, Williams LT: The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989-991, 1992.
 58. Terman BI, Vermazen MD, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. *Biochem Biophys Res Commun* 34:1578-1586, 1992.
 59. Millauer B, Witzigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A: High affinity binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835-846, 1993.
 60. Quinn T, Peters KG, deVries C, Ferrara N, Williams LT: Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 90:7533-7537, 1993.
 61. Shibuya M, Yamaguchi S, Yamane A, Ikada T, Tojo T, Matsushime H, Sato M: Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (*flt*) closely related to the *fms* family. *Oncogene* 8:519-527, 1990.
 62. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB: Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* 6:519-524, 1991.
 63. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR: A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to *c-kit*. *Proc Natl Acad Sci USA* 88:9026-9030, 1991.
 64. Kendell RL, Thomas KA: Inhibition of vascular endothelial growth factor by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* 90:10705-10709, 1993.
 65. Peters KG, deVries C, Williams LT: Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci USA* 90:8915-8919, 1993.
 66. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin C-H: Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269:26988-26995, 1994.
 67. Senger D, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 46:5269-5275, 1986.
 68. Rosenthal R, Megyesi JF, Henzel WJ, Ferrara N, Folkman J: Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. *Growth Factors* 4:53-59, 1990.
 69. Takahashi A, Sasaki H, Kim SJ, Tobisu K, Kakizoe T, Tsukamoto T, Kumamoto Y, Sugimura T, Tarada M: Markedly increased amounts of messenger RNA for VEGF and PIGF in renal cell carcinoma associated with angiogenesis. *Cancer Res* 54:4233-4237, 1994.
 70. Olson TA, Mohanraj D, Carson LF, Ramakrishnan S: Vascular permeability factor gene expression in normal and neoplastic human ovaries. *Cancer Res* 54:276-280, 1994.
 71. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 53:4727-4735, 1993.
 72. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth is a potential tumour angiogenesis factor in vivo. *Nature* 359:845-847, 1992.
 73. Phillips HS, Armanini M, Stavrou D, Ferrara N, Westphal M: Intense focal expression of vascular endothelial growth factor mRNA in human intracranial neoplasms: Association with regions of necrosis. *Int J Oncol* 2:913-919, 1993.
 74. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, Robertson JT, Ali IU, Oldfield EH: Expression of the vascular permeability/vascular endothelial growth factor gene in central nervous system neoplasms. *J Clin Invest* 91:153-159, 1993.
 75. Toi M, Hoshima S, Takayanagi T, Tominaga T: Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 85:1045-1049, 1994.

76. Ferrara N, Winer J, Burton T, Rowland A, Siegel M, Phillips HS, Terrell T, Keller G-A, Levinson AD: Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells. *J Clin Invest* 91:160-170, 1993.
77. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A: Glioblastoma growth is inhibited in vivo by a negative dominant Flk-1 mutant. *Nature* 367:576-579, 1994.
78. Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95:1789-1797, 1995.
79. Altman RJ, Mesiano S, Ferrara N, Jaffe RB: Vascular endothelial growth factor is essential for human ovarian carcinoma growth in vivo. Submitted.
80. Patz A: Studies on retinal neovascularization. *Invest Ophthalmol Vis Sci* 19:1133-1138, 1980.
81. Michaelson IC: The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. *Trans Ophthalmol Soc UK* 68:137-180, 1948.
82. Meyer-Schwickerath R, Pfeiffer A, Blum WF, Freyberger H, Klein M, Losche C, Rollman R, Schatz H: Vitreous levels of the insulin-like growth factors I and II, and the insulin-like growth factor binding proteins 2 and 3 increase in neovascular disease. *J Clin Invest* 92:2620-2625, 1993.
83. Hannehan A, deJuan E, Lutti GA, Fox GM, Schiffer S, Hjelmeland LM: Altered distribution of basic fibroblast growth factor in diabetic retinopathy. *Arch Ophthalmol* 109:1005-1011, 1991.
84. Adamis AP, Miller JW, Bernal M-T, D'Amico D, Folkman J, Yeo T-K, Yeo K-T: Increased vascular endothelial growth factor in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 118:445-450, 1994.
85. Miller JW, Adamis AP, Shima DT, d'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, Yeo T-K, Yeo K-T: Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular neovascularization in a primate model. *Am J Pathol* 145:574-584, 1994.
86. Koch AE, Harlow LA, Haines GK, Amento EP, Unemori EN, Wong W-L, Pope RM, Ferrara N: Vascular endothelial growth factor: a cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 152:4149-4156, 1994.
87. Fava RA, Olsen NJ, Spencer-Green G, Yeo T-K, Yeo K-T, Berse B, Jackman RW, Senger DR, Dvorak HF, Brown LF: Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid arthritis. *J Exp Med* 180:340-346, 1994.
88. Fassbender HJ, Simling-Annefeld M: The potential aggressiveness of synovial tissue in rheumatoid arthritis. *J Pathol* 139:399-406, 1983.
89. Hamilton J: Hypothesis: In vitro evidence for the invasive and tumor-like properties of the rheumatoid pannus. *J Rheumatol* 10:845-851, 1976.
90. Thompson RW, D'Amore PA: Recruitment of growth and collateral circulation. In: Zelenock GB, D'Alecy LG, Fantone JC III, Shlafer M, Stanley JC (eds) *Clinical Ischemic Syndromes: Mechanisms and Consequences of Tissue Injury*. Mosby, St. Louis, 1990, pp 117-134.
91. Graor RA, Gray BH: Interventional treatment of peripheral vascular disease. In: Young JR, Graor RA, Olin JW, Bartholomew JR (eds) *Peripheral Vascular Diseases*. Mosby, St. Louis, 1991, pp 111-133.

Critical Determinants of Neoplastic Angiogenesis

Isaiah J. Fidler, DVM, PhD, Rakesh K. Singh, PhD, Junya Yoneda, MD, Rakesh Kumar, PhD, Lei Xu, MD, Zhongyun Dong, MD, PhD, Diane R. Bielenberg, PhD, Marya McCarty, BS, Lee M. Ellis, MD, *Houston, Texas*

The major cause of death from cancer is metastases that are resistant to conventional therapy. Several reasons account for the failure to treat metastases. First, most tumors are biologically heterogeneous and contain multiple cell populations with diverse properties that include sensitivity to various cytotoxic agents and ability to invade and produce metastasis.^{1,2} Metastases can be clonal in origin, and different metastases can originate from different progenitor cells. The metastases can be located in different lymph nodes and different organs, and the specific organ environment can influence the biologic behavior of metastatic cells, including their response to therapy.^{1,3} Clearly, there is an urgent need to develop more effective regimens against disseminated cancer. A better understanding of the molecular mechanisms that regulate the process of metastasis and of the complex interactions between the metastatic cells and various host factors can provide the necessary biologic foundation for the design of more effective therapy.

THE PROCESS OF ANGIOGENESIS

One crucial step in the continuous growth of tumors and the development of metastasis is the induction of vasculature to tumors.⁴⁻⁸ A tumor mass that is < 1.0 mm in diameter can receive oxygen and nutrients by diffusion. Any increase in tumor mass requires the proliferation and morphogenesis of vascular endothelial cells, that is, angiogenesis.⁴ The process of angiogenesis consists of multiple, sequential, and interdependent steps. It begins with local degradation of the basement membrane surrounding capillaries, which is followed by invasion of the surrounding stroma by the underlying endothelial cells in the direction of the angiogenic stimulus. Endothelial cell migration is accompanied by the proliferation of endothelial cells, which are then organized into three-dimensional structures to form new capillary tubes⁵ (Fig. 1).

The onset of angiogenesis involves a change in the local equilibrium between proangiogenic and antiangiogenic molecules.⁹ Some of the common proangiogenic factors are basic fibroblast growth factor (bFGF), which induces the proliferation of a variety of cells and also stimulates endothelial cells to migrate, to increase production of proteases, and to undergo morphogenesis;^{7,10-12} vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), which induces the proliferation of endothelial cells, increases vascular permeability, and induces production of urokinase plasminogen activator (uPA) by endothelial cells;¹³⁻¹⁵ interleukin-8 (IL-8), a chemoattractant cytokine produced by a variety of tissues and blood cells that attracts and activates neutrophils in inflammatory regions and is angiogenic in rat cornea assay;¹⁶⁻¹⁸ platelet-derived endothelial cell growth factor (PD-ECGF), which stimulates endothelial cell DNA synthesis and chemotaxis and induces production of FGFs;¹⁹ hepatocyte growth factor (HGF), or scatter factor, a heparin-binding glycoprotein that increases cell migration, cell invasion, and the production of proteases as well as stimulation of angiogenesis;^{20,21} and platelet-derived growth factor (PDGF), a potent growth factor for mesenchymal and glial cells.²² The role of PDGF in angiogenesis at present is not clear but it has been shown to be angiogenic on chick chorioallantoic membrane²³ and possibly angiogenic during placental development and tumor growth.^{24,25}

Recently, we studied some of the mechanisms by which the different proangiogenic molecules regulate different steps in the process of angiogenesis.²⁶ Many growth factors can induce cell division and enhance cell survival. In the absence of serum, endothelial cells cannot survive in culture.²⁶⁻²⁸ Hence, culture conditions using serum-free media with different concentrations of cytokines can determine the ability of the various cytokines to enhance survival. Our studies conclude that VEGF/VPF and bFGF are both effective survival factors, in agreement with published reports.²⁶

The most common in vitro assay for angiogenesis is the induction of endothelial cell proliferation.¹²

From the Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

Reprint requests: Isaiah J. Fidler, DVM, PhD, Department of Cancer Biology, Box 173, 1515 Holcombe Boulevard, Houston, TX 77030.

Copyright © 2000 Jones and Bartlett Publishers.

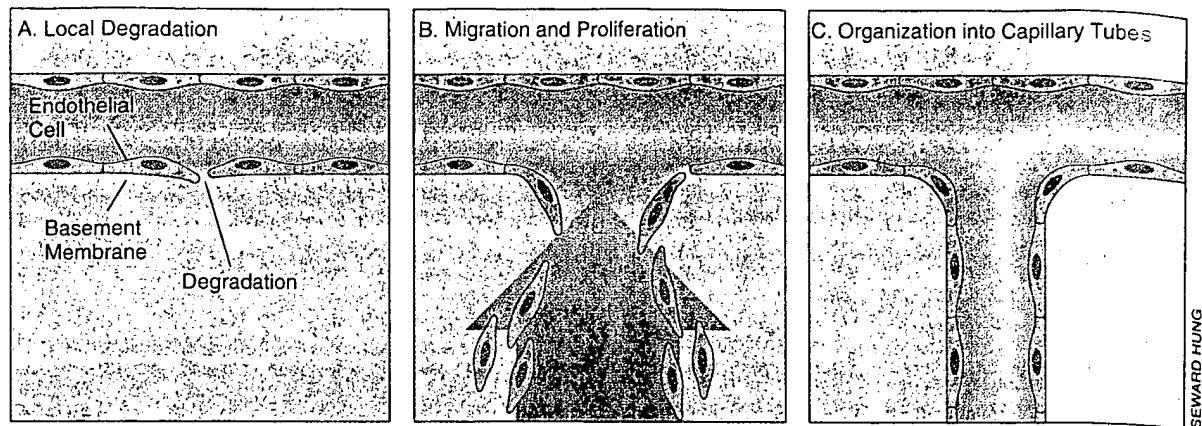


Figure 1 The process of angiogenesis. The process begins with degradation of the capillary basement membrane by metalloproteinases (A). Endothelial cells then migrate into the stroma following a gradient of angiogenic stimuli and proliferate within the stroma (B). Organization of endothelial cells into three-dimensional structures forming new capillary tubes completes the process (C).

We concluded that, among the cytokines tested, bFGF is the most potent mitogen. The initial discovery of VEGF by Dvorak and coworkers was based on its ability to increase vascular permeability; the factor was therefore called VPF.²⁹ The VPF gene was later cloned by Ferrara et al and named VEGF.³⁰ In our experiments, VEGF/VPF also induced division of endothelial cells, albeit to a much lower degree than did bFGF, in agreement with earlier reports.³¹

Increased proteolytic activity is critical for angiogenesis because degradation of the extracellular matrix (ECM) allows endothelial cells to invade and migrate. Although several enzymes have been found to degrade ECM, the most widely implicated enzymes involved in angiogenesis include matrix metalloproteinases, such as interstitial collagenase (MMP-1) and gelatinases (MMP-2 and -9), serine protease, and plasminogen activator.^{8, 32, 33} Exogenous and endogenous bFGF up-regulates MMP-2 in murine melanoma cells³⁴ and enhances the expression of MMP-1 and uPA in human umbilical vein endothelial cells (HUVEC). Although both bFGF and VEGF/VPF induce uPA in microvascular endothelial cells,³⁵ we did not find up-regulation of uPA mRNA in HUVEC by VEGF/VPF. These differences may be attributable to the different origin of endothelial cells used (microvascular versus umbilical) or to differences in culture conditions.

Another important step in angiogenesis is migration of endothelial cells, and bFGF, VEGF/VPF, and HGF are chemotactic to HUVEC.³¹ Our data suggest that bFGF and VEGF/VPF are chemotactic for endothelial cells, whereas HGF enhances the random migration of these cells, consistent with its property as a scatter factor.^{36, 37}

To complete the process of angiogenesis, newly formed endothelial cells must organize into capillaries. The measurement of this morphogenesis is based on the *in vitro* formation of multicellular or

tubelike structures that resemble microvascular sprouts or networks, which is facilitated by placing the endothelial cells in contact with components of the ECM.³⁸ We cultured HUVEC on top of gelled basement membrane matrix (Matrigel) in the presence of different concentrations of angiogenic factors. IL-8, VEGF/VPF, and bFGF all induced the organization of HUVEC into an extensive network that resembled a capillary mesh.

Besides modulating endothelial proliferation, migration, and morphogenesis, growth factors can also modify vascular permeability, as demonstrated by VEGF/VPF.¹⁵ Increased permeability of blood vessels during angiogenesis possibly facilitates the extravasation of proteins and, thus, the formation of ECM for cell migration.¹⁵ Acute local administration of VEGF has been shown to transiently increase vascular permeability³⁹ through activation of vesicular-vacuolar organelles present in the cytoplasm of endothelial cells⁴⁰ and through the induction of interendothelial cell gaps and endothelial fenestration.⁴¹ The expression of VEGF/VPF by endothelial cells and other cell types is regulated by many factors, hypoxia being the foremost.⁴² Because hypoxia can produce irreversible tissue damage, the induction of vascular permeability is the acute response, and its regulation by VEGF/VPF demonstrates the unique importance of this response in maintaining the functional integrity of the microvasculature. Of all the cytokines tested, only VEGF/VPF increased the permeability of HUVEC, thus confirming the vascular permeability noted on the initial discovery of this factor.²⁹

Different cytokines may have indirect angiogenic activity. For example, lymphoid cells have been shown to induce angiogenesis in both physiological^{43, 44} and pathological^{45, 46} conditions. Cytokines that activate lymphoid cells to release angiogenic molecules can be classified as angiogenic themselves.⁴⁷ Second, many studies examining the

expression of angiogenesis-related genes in neoplasms, for example, angiogenic molecules and their receptors, have found an abundance of VEGF/VPF and its receptors⁴⁸⁻⁵⁰ contrasted with rare expression of bFGF and its receptors.^{51, 52} Several factors may account for this finding. First, the turnover time of capillary endothelial cells exceeds 1000 days.^{53, 54} Thus, cell division, which takes only hours, is a rare event, whereas continuous functions that depend on permeability are common. Second, the demonstrations that sparse cultures of actively dividing cells express high amounts of bFGF,⁵⁵ whereas confluent nondividing cells express high amounts of VEGF/VPF,⁵⁶ coupled with the finding that bFGF is expressed on the periphery of human neoplasms and not in the center of the lesions,⁵⁷ may explain this discrepancy. There are some differences in endothelial cells originating from large vessels (HUVEC) and microvessels⁵⁸⁻⁶⁰ as well as in endothelial cells from different organs.⁶¹⁻⁶⁴

■ REGULATION OF ANGIOGENESIS BY THE TUMOR MICROENVIRONMENT

Our laboratory has shown that expression of bFGF by tumor cells depends also on the site of implantation. When human renal cell carcinoma (HRCC) cells were implanted in different organ microenvironments in nude mice, the expression of bFGF was 10 to 20 times higher in those tumors implanted in the kidney than in those implanted in the subcutaneous tissues.⁶⁵ The kidney tumors were more highly vascularized than the tumors implanted in the subcutis. In sharp contrast, the expression of interferon- β (IFN- β) was high in epithelial cells and fibroblasts surrounding the subcutaneous tumors, whereas no IFN- β was found in or around HRCC tumors growing in the kidney. The parental cell line and metastatic clone also differed in bFGF expression. The alteration in bFGF level by the site of implantation was the result of adaptation to the organ microenvironment, as demonstrated when the cells were reestablished in culture and their bFGF content returned to its previous *in vitro* concentration after 4 weeks.⁶⁵

Expression of bFGF in HRCC is cell density-dependent.⁶⁶ By *in situ* mRNA hybridization (ISH) and northern blot analysis, we found an inverse correlation between increasing cell density and bFGF expression.⁶⁶ Fluorescence-activated cell sorting, immunohistochemistry, and enzyme-linked immunosorbent assay confirmed this finding at the protein level. Tumor cells harvested from dense cultures (low bFGF expression) and then plated under sparse conditions expressed high amounts of bFGF. Similar data were obtained by using endothelial cells. The effect was not mediated by soluble factors released into the culture medium. The *in vivo* manifestation of cell density-dependent regulation is

likely to be found by differences in gene expression in the center of a tumor versus at the periphery or leading edge.^{67, 68}

The expression of bFGF in surgical specimens of human colon carcinoma (HCC) was determined by ISH and northern blot analysis. ISH analysis revealed that the concentration of bFGF was markedly higher in Duke's stage C or D tumors than in Duke's stage B tumors. Northern blot hybridization did not detect mRNA transcripts for bFGF. However, analysis by ISH revealed that bFGF was overexpressed at the periphery of the tumor (leading edge), where the cells were rapidly dividing. This observation confirms that tumors are heterogeneous for cells having various degrees of expression of invasion and metastasis-related genes and that a subpopulation of cells within a tumor can give rise to distant metastasis. In a follow-up study with colon cancer patients, bFGF expression was found to be highest in the primary tumors of patients who presented with metastatic disease. This study identified patients who appeared to be free of metastasis at the time of initial surgery (Duke's stage B) and yet developed distant metastasis at a later date; these patients had relatively high bFGF expression along with increased expression of other metastasis related genes, especially at the invasive edge of the tumor.^{67, 68}

The production of angiogenic molecules (e.g., VEGF, bFGF, and IL-8) by melanoma cells is regulated by complex interactions with keratinocytes in the skin.⁶⁹ Recent reports from our laboratory show that IL-8 is an important molecule in melanoma growth and progression. Not only did constitutive expression of IL-8 directly correlate with the metastatic potential of human melanoma cells, but also IL-8 induced proliferation, migration, and invasion of endothelial cells and, hence, neovascularization.^{17, 70} Several organ-derived cytokines (produced by inflammatory cells) are known to induce expression of IL-8 in normal and transformed cells.⁶⁹ Because IL-8 expression in melanocytes and melanoma cells can be induced by inflammatory signals, we analyzed whether specific organ microenvironments could influence the expression of IL-8. Melanoma cells were implanted into the subcutis and the spleen of athymic nude mice (to produce liver metastasis) and were supplied intravenously (to produce lung metastasis). Subcutaneous tumors, lung lesions, and liver lesions expressed high, intermediate, and no IL-8 mRNA and protein, respectively.⁷¹ Melanoma cells established from the tumors growing *in vivo* exhibited similar amounts of IL-8 mRNA transcripts as continuously cultured cells, thus demonstrating that the differential expression of IL-8 was the result of the selection of a subpopulation of cells⁷¹ (Fig. 2).

IL-8 expression can be up-regulated by coculturing melanoma cells with keratinocytes (skin) and in-

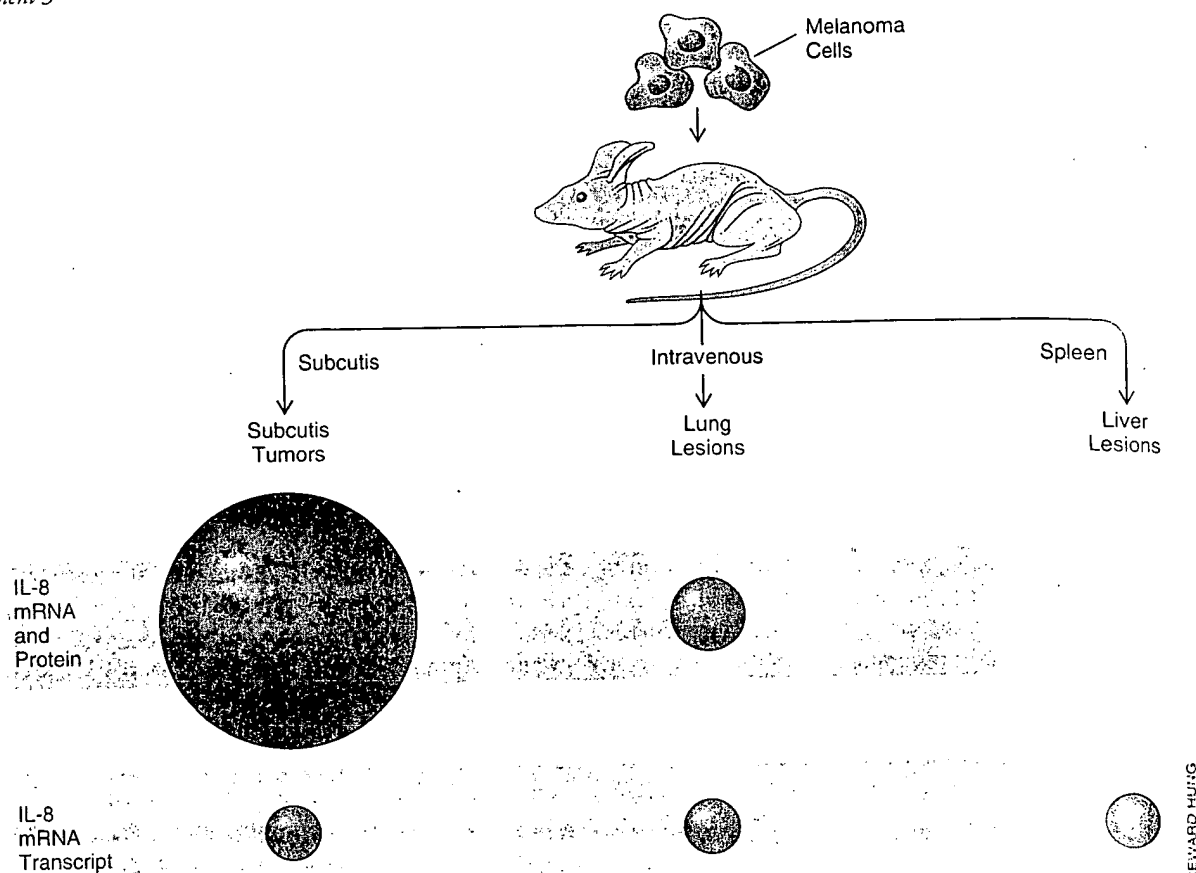


Figure 2 The organ microenvironment regulates expression of IL-8. Melanoma cells were implanted at each of three different sites in nude mice: subcutis, spleen (liver metastasis), and intravenous (lung metastasis). Expression of IL-8 (mRNA and protein) was high in subcutis tumors, intermediate in lung lesions, and absent from liver lesions. Transcripts for mRNA were similar in cells from all three sites, so cell selection was not responsible for the differences in expression.

hibited by coculturing melanoma cells with hepatocytes (liver). We also investigated the effects of two cytokines produced by keratinocytes (IL-1, IFN- β) and two cytokines produced by hepatocytes (tumor growth factors α and β : TGF- α , TGF- β) on the regulation of IL-8 in human melanoma cells. IL-1 up-regulated the expression of IL-8 in human melanoma cells at both the mRNA and protein levels in a dose- and time-dependent manner in the presence of de novo protein synthesis.¹⁷ IFN- β did not affect constitutive IL-8 mRNA and protein production in human melanoma cells, but it did block the induction of IL-8 by IL-1.¹⁷ TGF- β inhibited the expression of IL-8, whereas TGF- α had no effect on IL-8 expression.

The expression of the common angiogenic molecule VEGF is increased in necrotic areas of human tumor.⁴⁸ In vitro studies have confirmed that VEGF expression is increased in response to hypoxia, probably because of increased transcription and increased mRNA stability.⁷² Treatment of cells with IL-1, IL-6, IL-8, TGF- β , PDGF, HGF, and bFGF can increase expression of VEGF.⁴⁹ VEGF expression is also regulated by certain oncogenes (*src* and *ras*) and tumor suppressor genes, such as *p53*.⁷³ The organ microenvironment also influences the expression of

VEGF/VPF. Human gastric cancer cells were implanted into orthotopic (stomach) and ectopic (subcutaneous) organs of nude mice. Tumors in the stomach were highly vascularized, expressed high amounts of VEGF, and grew more rapidly than did the subcutaneous tumors. In addition, metastasis occurred only from the tumors implanted in the stomach.^{74, 75}

■ LYMPHOID-MEDIATED ANGIOGENESIS

Angiogenesis is essential to homeostasis, and its regulation by lymphoid cells, such as T-lymphocytes, macrophages, and mast cells, is well recognized.^{43-47, 76-83} A local inflammatory reaction consisting of T-lymphocytes and macrophages is often associated with invasive cutaneous melanoma, and an intense inflammatory reaction is often associated with increased risk of metastasis, suggesting that angiogenesis induced by inflammation may contribute to tumor progression and metastasis.

Immunological mechanisms involved in physiological angiogenesis occur subsequent to wound healing.⁴³ Systemic chemotherapy has been shown to retard the process of wound healing, possibly by decreased immune response; whether this is mediated by inhibition of angiogenesis is not clear.⁸⁰ When

investigating the role of tumor vascularization and its effect on tumor growth in immunosuppressed mice, we found the growth of weakly immunogenic B16 melanoma to be slower in myelosuppressed mice than in control mice.⁴⁶ Further evidence implicating myelosuppression in the retardation of tumor growth and vascularity was obtained from doxorubicin (DXR)-pretreated animals injected with normal spleen cells 1 day before tumor challenge. Tumor growth in these mice was comparable with that in control mice.⁴⁶ Similar results were obtained in athymic mice, suggesting that the tumor vascularization observed in DXR-treated mice reconstituted with normal spleenocytes was not mediated solely by T-lymphocytes. Because reconstitution with spleen cells enhanced vascularization of the B16 tumors, the results suggest that myelosuppressive chemotherapeutic drugs, DXR, for example, can inhibit host-mediated vascularization and support the concept that developing tumors can usurp homeostatic mechanisms to their advantage.³

The role of infiltrating cells in angiogenesis of human colon cancer has recently been reported by Takahashi et al.⁷⁴ They observed high expression of PD-ECGF in infiltrating cells, which were mostly macrophages and lymphocytes, and very little expression of PD-ECGF in the cancer epithelium. The intensity of staining for PD-ECGF in infiltrating cells correlated with the density of blood vessels, suggesting the involvement of these cells in the angiogenesis of HCC.

Macrophages have been recognized for several years as important angiogenesis effector cells.^{43, 45, 47, 84, 85} They may influence new capillary growth by several different mechanisms. First, macrophages produce factors that act directly to influence angiogenesis-linked endothelial cell functions. In vitro studies have shown that macrophages produce > 20 molecules that reportedly influence endothelial cell proliferation, migration, and differentiation in vitro^{45, 86} and are potentially angiogenic in vivo. A second mechanism by which macrophages might modulate angiogenesis is by modifying the ECM. The composition of the ECM has been shown to influence endothelial cell shape and morphology dramatically and may profoundly influence new capillary growth.⁸⁶⁻⁸⁸ Macrophages can influence the composition of the ECM either through the direct production of ECM components or through the production of proteases, which effectively alter the structure and composition of the ECM.⁴³ A third mechanism is the production of substances that suppress angiogenesis. Macrophages have been shown to express the angiogenesis inhibitor thrombospondin-1 when treated with the chemopreventive agent retinoic acid.^{86, 89-91}

Recently, we examined the mechanism for generation of angiostatin, an angiogenesis inhibitor iso-

lated from plasma of mice bearing Lewis lung carcinoma (3LL).⁹² Our results demonstrate that the generation of angiostatin by the subcutaneous tumors requires the presence of macrophages and is directly correlated with their metalloelastase activity.⁹³ The addition of plasminogen to 3LL cells cultured in vitro did not result in generation of angiostatin, whereas the addition of plasminogen to cocultures of macrophages and 3LL cells did. Elastase activity in macrophages was up-regulated by the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF secreted by 3LL cells markedly enhanced the production of elastase by macrophages and hence the generation of angiostatin from plasminogen.⁹³ These data suggest that elastase released from tumor-infiltrating macrophages is responsible for the angiostatin production in this tumor model and for the angiogenesis-inhibiting role of macrophages.

■ MOLECULAR DETERMINANTS OF ANGIOGENESIS IN CUTANEOUS HEMANGIOMAS

Infantile cutaneous hemangiomas represent a unique form of pathological angiogenesis in which endothelial cell tumors grow rapidly in the first year of life (proliferative phase), followed by a slow regression during the next 5 years (involuting phase) and an eventual involution or complete regression (involved phase) by age 10 to 15 years.⁹⁴ Chronic daily systemic treatment with IFN- α has been shown to accelerate the involution of fatal hemangiomas.⁹⁵⁻⁹⁹ To determine whether the progression and involution of infantile cutaneous hemangiomas were associated with overexpression of proangiogenic molecules or the lack of antiangiogenic molecules, we analyzed a large number of hemangioma specimens by immunohistochemistry. We found that proliferating hemangiomas expressed bFGF and VEGF/VGF but not IFN- β (mRNA and protein).¹⁰⁰ A surprising finding was that the epidermis directly overlying proliferating hemangiomas was hyperplastic, whereas the epidermis overlying involuted hemangiomas or the epidermis from an unaffected site was not (Fig. 3). The hyperplastic epidermis expressed bFGF, VEGF/VPF, and IL-8 but not IFN- β , whereas the normal epidermis expressed both positive and negative angiogenic molecules. These data raised the possibility that the proliferating hemangiomas induced hyperplasia in the surrounding normal tissues (epidermis), leading to production of bFGF and VEGF/VPF, but not IFN- β ,¹⁰⁰ supporting the concept that neoplastic cells subvert and usurp host homeostatic mechanisms for their growth advantage.

To study the relationship between hemangiomas and the microenvironment, we developed an in vivo model for epidermal hyperplasia and angiogenesis, using UV-B irradiation of mice.¹⁰¹ Mice exposed to 10 kJ/m² UV-B developed epidermal hyperplasia ac-

Spleen
↓
Liver
Lesions



SEWARD HUNG

three differ-
ent mRNA
for
expression.

were im-
munopositive (sub-
types in the
expressed high
than did
metastasis
ed in the

and its reg-
ulation by
phocytes,
regulated.⁴³⁻⁴⁷
regulation of T-
cells associated
with intense
angiogenesis
with in-
creased angio-
gene-
sis to tu-

n physio-
logical wound
healing shown
possibly by
its mediators.⁸⁰ When

accompanied by angiogenesis and telangiectasia during the first week following irradiation; however, these slowly subsided over the following weeks. The first striking event after UV-B irradiation was the increase in production of bFGF in the keratinocytes of the epidermis.¹⁰¹ The increase in bFGF preceded or at least coincided with the division of epidermal cells, as recognized by immunohistochemical staining with antibodies to proliferating cell nuclear antigen. Marked hyperplasia and angiogenesis immediately followed. The expression of VEGF/VPF was slightly increased by day 5. Most interesting, the expression of IFN- β in the epithelium decreased with epidermal hyperplasia but was reexpressed as the hyperplasia and angiogenesis subsided.¹⁰²

■ ANTIANGIOGENIC ACTIVITY OF INTERFERON- β

The IFN family consists of three major glycoproteins that exhibit species specificity: leukocyte-derived IFN- α , fibroblast-derived IFN- β , and immune cell-produced IFN- γ . Although IFN- α and IFN- β share a common receptor (the type I IFN receptor) and induce a similar pattern of cellular responses, certain cellular reactions can be stimulated only by IFN- β , probably by the phosphorylation of a receptor-associated protein that is uniquely responsive to IFN- β .¹⁰³ In addition to their well-recognized activity as antiviral agents, IFNs regulate multiple biological activities such as cell growth,¹⁰⁴ differentiation,¹⁰⁵ oncogene expression,^{107, 108} host immunity,¹⁰⁹⁻¹¹¹ and tumorigenicity.¹¹²⁻¹¹⁷ IFNs can also inhibit several steps in the angiogenic

process. IFN has antiproliferative properties, especially on tumor cells,¹¹⁸⁻¹²⁰ an effect that has also been demonstrated on endothelial cells *in vitro*. IFN- α can inhibit FGF-induced endothelial proliferation,¹²¹ and IFN- γ can inhibit endothelial proliferation.¹²² IFN- α and IFN- γ have been shown to be cytostatic to human dermal microvascular endothelial cells¹²³ and human capillary endothelial cells.¹²⁴

Systemic therapy using recombinant IFNs produces antiangiogenic effects in vascular tumors, including hemangioma,⁹⁴⁻⁹⁹ Kaposi's sarcoma,¹²⁵⁻¹²⁸ melanoma,¹²⁹ basal cell and squamous cell carcinoma,¹³⁰ and bladder carcinoma.¹³¹ These tumors have also been documented as producing the high amounts of bFGF that are often detectable in the urine or serum of these patients.¹³²⁻¹³⁴ These findings, along with our *in vivo* observations, prompted us to investigate whether IFNs could modulate the expression of the angiogenic molecule bFGF. We found that IFN- α and IFN- β but not IFN- γ down-regulated the expression of bFGF mRNA and protein in HRCC as well as in human bladder, prostate, colon, and breast carcinoma cells.¹³⁵ The inhibitory effect of IFN- α and - β on bFGF expression was cell density-dependent and was independent of the antiproliferative effects of IFNs.^{70, 135} We also confirmed that IFN can inhibit bFGF production in an *in vivo* model system. Systemic administration of human IFN- α decreased the *in vivo* expression of bFGF, decreased blood vessel density, and inhibited tumor growth of a human bladder carcinoma implanted orthotopically in nude mice.¹³⁶

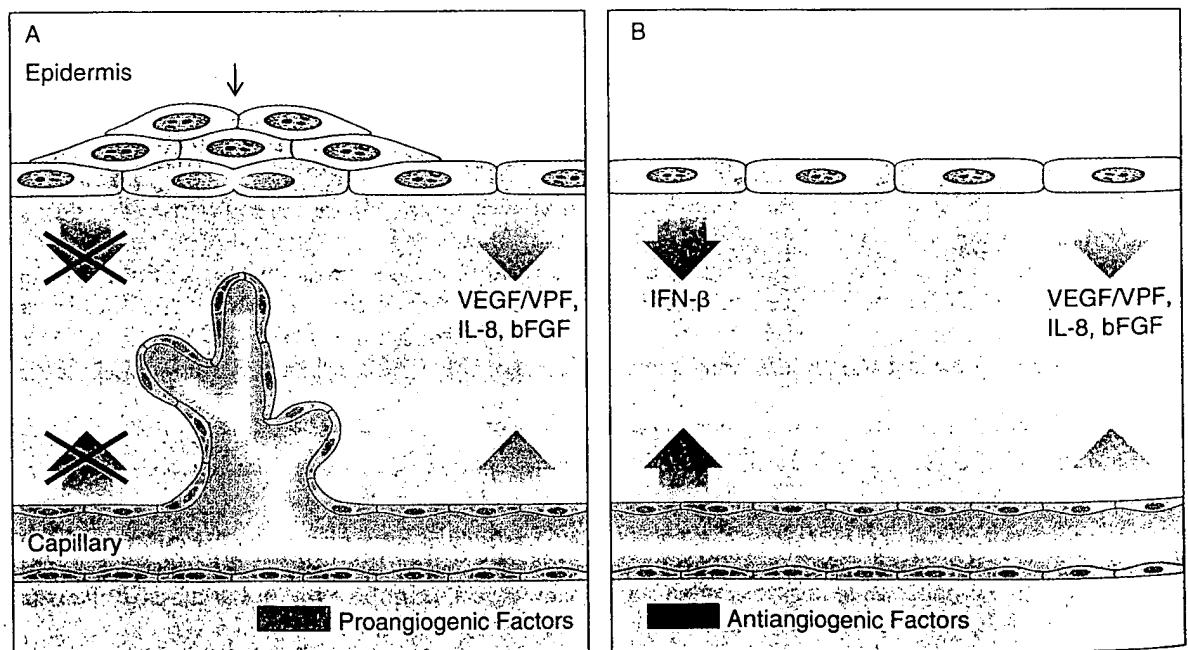


Figure 3 Molecular determinants of angiogenesis in cutaneous hemangiomas. Specimens were examined by immunohistochemistry. Epidermis overlaying proliferating hemangiomas (A) was hyperplastic (arrow) and expressed only proangiogenic bFGF, VEGF/VPF, and IL-8, but not antiangiogenic IFN- β mRNA and protein. In contrast, epidermis from unaffected sites or involuted hemangiomas (B) was not hyperplastic and expressed both proangiogenic and antiangiogenic molecules.

The antiangiogenic effect of IFNs cannot be explained solely on the basis of inhibition of endothelial cell proliferation. For example, IFN- α/β can also inhibit the endothelial cell migration step of angiogenesis.^{137, 138} Subcutaneous injection of IFN- α/β adjacent to a wound delayed the healing process by inhibiting the proliferation, migration, and invasion of capillary buds, fibroblasts, and epithelial cells.^{139, 140} IFN- α/β injected intratumorally or peritumorally into tumor cells resistant to the antiproliferative effects of IFN damages blood vessels, leading to ischemia and necrosis.^{141, 142} Moreover, as we reported, IFN- α/β can affect the expression of several angiogenic factors, including bFGF,^{70, 136} IL-8,¹⁴² and collagenase type IV.^{143, 144} (Fig. 4).

■ CONSTITUTIVE EXPRESSION OF IFN- β IN DIFFERENTIATED EPITHELIAL CELLS EXPOSED TO ENVIRONMENTAL STIMULI

The body's first line of defense against external challenge is the epithelial cells that line the skin and the respiratory, digestive, and genitourinary tracts. Inasmuch as IFN- β participates in host defense against viral, bacterial, and parasitic infections and tumors, we hypothesized that this secreted protein might be expressed in various murine epithelial cell types that line portals of entry to the body. We used immunohistochemistry and ISH techniques to measure IFN- β expression in the various epithelial cell types and in internal murine organs sheltered from environmental stimuli. The epithelial cell types lining the skin, digestive tract, urinary tract, reproductive tract, and upper respiratory tract constitutively expressed IFN- β . Specifically, all differentiated epithelial cells that are at risk of environmental exposure expressed IFN- β (protein and mRNA), except for the ciliated epithelial cells lining the lower respiratory tract. Epithelial cells of internal organs that are not directly exposed to external pathogens did not express IFN- β .¹⁴⁵

Because IFN- β regulates cell replication and function, we determined whether the expression of IFN- β by epithelial cells correlated with terminal differentiation.¹⁴⁶ ISH analysis and immunohistochemical staining of formalin-fixed, paraffin-embedded specimens of normal human and murine epidermis and human and murine skin tumors of epithelial origin revealed that only differentiated, nondividing cells of the epidermis expressed IFN- β protein. Keratinocyte cultures established from the epidermis of 3-day-old mice were maintained under conditions permitting continuous cell division or induction of differentiation. Continuously dividing cells did not produce IFN- β , whereas nondividing differentiated cells expressing keratin 1 did. Growth-arrested, undifferentiated keratinocytes also expressed IFN- β protein. Neutralizing IFN- β in the culture medium inhibited differentiation, but adding exogenous IFN-

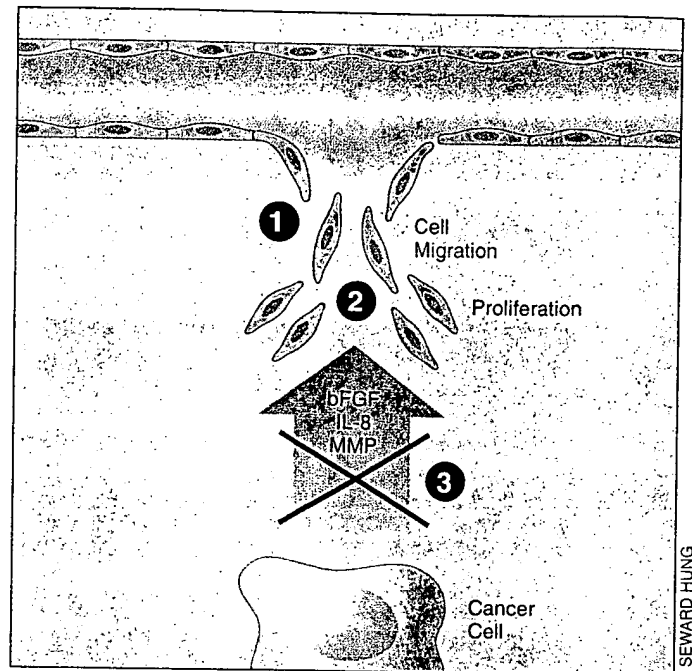


Figure 4 Antiangiogenic activity of IFN- α/β . IFNs can inhibit a number of steps in the angiogenic process, including endothelial cell migration (1) and proliferation (2), and the expression of the proangiogenic factors bFGF, IL-8, and matrix metalloproteinase (MMP; 3).

β did not stimulate differentiation. These data indicate that IFN- β is produced by growth-arrested, terminally differentiated keratinocytes.^{145, 146}

■ THERAPY OF HUMAN OVARIAN CANCER BY TRANSFECTION WITH THE MURINE IFN- β GENE: ROLE OF MACROPHAGE-INDUCIBLE NITRIC OXIDE SYNTHASE

Our laboratory recently demonstrated that the local sustained production of murine IFN- β could inhibit the growth of human ovarian cancer cells in the peritoneal cavity of nude mice.¹⁴⁷ Human ovarian tumor Hey-A8 cells transfected with mIFN- β or a control neomycin-resistance vector grew well in culture. Tumor cells were injected into the peritoneal cavity or under the subcutis of nude mice. Parental (wild-type) or control transfected cells produced large tumors, whereas mIFN- β -transfected cells did not produce any tumors. The IFN- β -transfected cells prevented the outgrowth of bystander parental, control-transfected cells, and another human ovarian tumor cell line, SKOV3i.p.1, in the peritoneal cavity of nude mice (Fig. 5). The IFN- β -transfected tumor cells stimulated a high amount of NO production in murine macrophages under both in vitro and in vivo conditions, and only the NO-producing macrophages exhibited antitumor activity.¹⁴⁷ Collectively, these results demonstrate that the local production of IFN- β can inhibit the in vivo growth of human ovarian cancer cells by up-regulating the expression of the inducible nitric oxide synthase (iNOS) gene in host macrophages.¹⁴⁸

F/VPF,
bFGF

SEWARD HUNG

mis

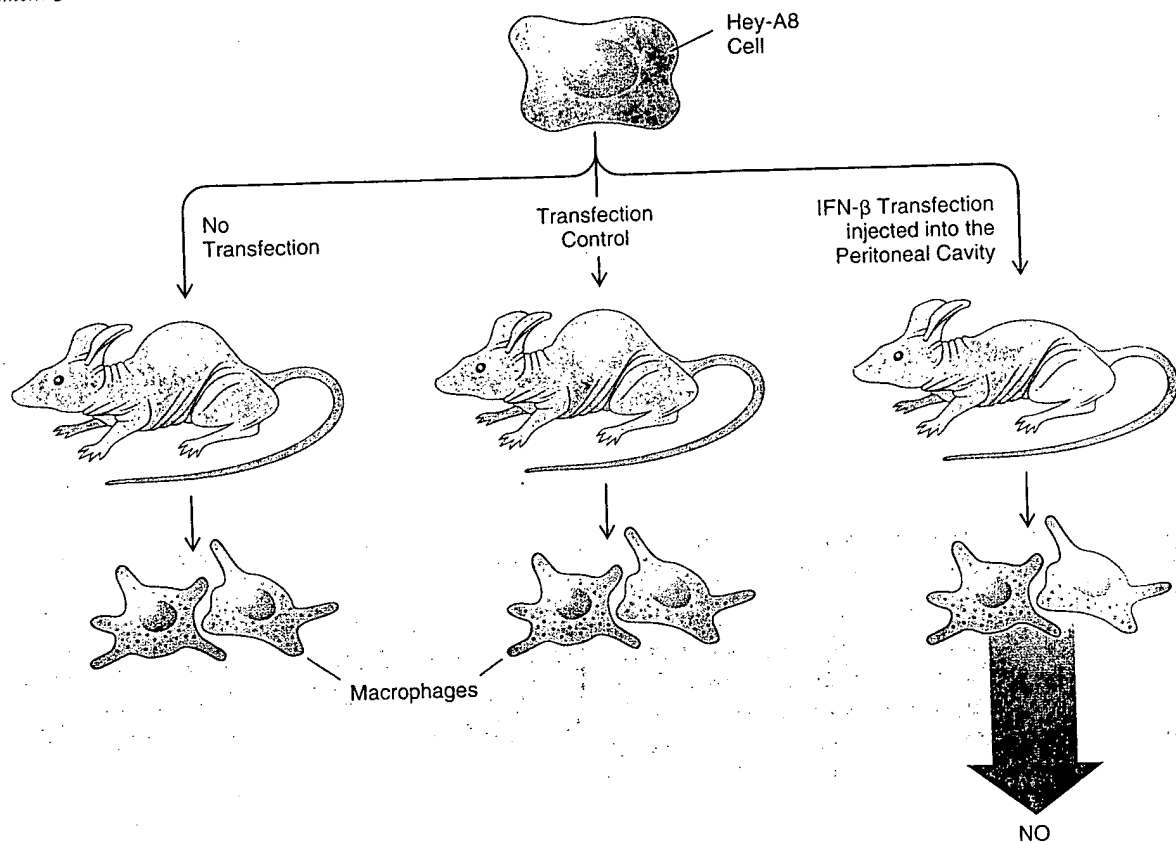


Figure 5. Therapy of human ovarian cancer by transfection with murine IFN- β . Human ovarian tumor Hey-A8 cells were transfected with mIFN- β or a control gene for neomycin resistance. Nude mice injected into the peritoneal cavity with the unaltered Hey-A8 or control-transfected cells grew large tumors, but those injected with IFN- β -transfected cells did not have tumors. Macrophages from mice injected with IFN- β -transfected cells produced large amounts of NO, but those from mice injected with the parental or control-infected cells did not.

■ SUPPRESSION OF ANGIOGENESIS, TUMORIGENICITY, AND METASTASIS BY HUMAN PROSTATE CANCER CELLS ENGINEERED TO PRODUCE IFN- β

We also determined whether the IFN- β could be used to suppress angiogenesis, tumor growth, and metastasis of human prostate cancer cells growing in the prostate of nude mice.¹⁴⁹ Highly metastatic PC-3M human prostate cancer cells were engineered to constitutively produce murine IFN- β subsequent to infection with a retroviral vector containing murine IFN- β cDNA. Parental (PC-3M-P), control vector-transduced (PC-3M-Neo), and IFN- β -transduced (PC-3M-IFN- β) cells were injected into the prostate (orthotopic) or subcutis (ectopic) of nude mice. PC-3M-P and PC-3M-Neo cells produced rapidly growing tumors and regional lymph node metastases, whereas PC-3M-IFN- β cells did not. PC-3M-IFN- β cells also suppressed the tumorigenicity of bystander nontransduced prostate cancer cells. PC-3M-IFN- β cells produced small tumors (3–5 mm in diameter) in nude mice treated with anti-asialo GM1 antibodies and in severe combined immunodeficient/Beige mice. Immunohistochemical staining revealed that PC-3M-IFN- β tumors were homogeneously infiltrated by macrophages, whereas

control tumors contained fewer macrophages at their periphery. Most tumor cells in the control tumors were stained positive by an antibody to proliferative cell nuclear antigen (PCNA); very few were positively stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL). In sharp contrast, PC-3M-IFN- β tumors contained fewer PCNA-positive cells and many TUNEL-positive cells. Staining with antibody against CD31 showed that the control tumors contained more blood vessels than did the PC-3M-IFN- β tumors. PC-3M-IFN- β cells were more sensitive to lysis mediated by NK cells in vitro or to cytotoxicity mediated by macrophages than control transduced cells. Conditioned medium from PC-3M-IFN- β cells augmented splenic cell-mediated cytotoxicity to control tumor cells, which could be neutralized by antibody against IFN- β . Collectively, the data suggest that the suppression of tumorigenicity and metastasis of PC-3M-IFN- β cells is the result of inhibition of angiogenesis and activation of host effector cells.¹⁴⁸

■ CONCLUSIONS

The angiogenesis within and surrounding neoplasms is regulated by the balance between proan-

giogen
and ar
statin,
cells, a
The ci
cells r
signal
their
all of
the tu
regula
enhar
for th
angio
anistr
sels.
of ho
from
Resto
of ar
mini
tion
duce
orth
whe
of h

■ A

Thi
Sup
CA
tion

■ F

1.

2.

3

4

5

6

giogenic molecules (e.g., bFGF, VEGF/VPF, IL-8) and antiangiogenic molecules (e.g., IFN, angiostatin, thrombospondin). Tumor cells, normal host cells, and leukocytes all contribute to angiogenesis. The cross talk between the tumor cells and stromal cells represents a complex network of extracellular signals, including cytokines and growth factors, their antagonists/inhibitors, and soluble receptors, all of which are released by and act on cells within the tumor microenvironment. The net effect is to regulate the proliferation of metastatic cells and to enhance the establishment of a new blood supply for the tumors. Tumor cells express more than one angiogenic molecule, suggesting a redundant mechanism for regulating the growth of new blood vessels. These factors may be regulated by different sets of host-derived cytokines. The absence of IFN- β from tumor beds is associated with angiogenesis. Restoring IFN provides an approach to the control of angiogenesis in neoplasms. Frequent systemic administrations of low-dose IFN- α or - β or introduction of the IFN- β gene into the tumor bed has produced substantial therapeutic results in preclinical orthotopic models. Clinical trials should determine whether this approach is also useful for the therapy of human cancers.

ACKNOWLEDGMENT

This work was supported in part by Cancer Center Support Core Grant CA16672 and grant R35-CA42107 from the National Cancer Institute, National Institutes of Health.

REFERENCES

- Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth GHA Clowes memorial award lecture. *Cancer Res* 1990;50:6130-6138.
- Radinsky R, Aukerman SL, Fidler IJ. The heterogeneous nature of metastatic neoplasms: relevance to biotherapy. In: Oldham RK, ed. *Principles of Cancer Biotherapy* (3rd ed). Boston: Kluwer Academic, 1998:16-38.
- Fidler IJ. Modulation of the organ microenvironment for the treatment of cancer metastasis [editorial]. *J Natl Cancer Inst* 1995;84:1588-1592.
- Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nat Med* 1995;1:27-31.
- Auerbach W, Auerbach R. Angiogenesis inhibition: a review. *Pharmacol Ther* 1994;63:265-311.
- Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994;79:185-188.
- Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987;235:444-447.
- Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991;64:327-336.
- Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353-364.
- Bouck N, Stellmach V, Hsu SC. How tumors become angiogenic. *Adv Cancer Res* 1996;69:135-174.
- Folkman J. *Seminars in Medicine of the Beth Israel Hospital*. Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 1995;333:1757-1763.
- Cockerill GW, Gamble JR, Vadas MA. Angiogenesis: models and modulators. *Int Rev Cytol* 1995;159:113-160.
- Leung ZW, Cachianes G, Kuang WJ et al. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246:1306-1309.
- Plate KH, Breier G, Weich HA et al. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature* 1992;359:845-848.
- Dvorak HF, Brown LF, Detmar M et al. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029-1039.
- Koch AE, Polverini PJ, Kunkel SL et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 1992;258:1798-1801.
- Singh RK, Gutman M, Radinsky R et al. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. *Cancer Res* 1994;54:3242-3247.
- Yoneda J, Kuniyasu H, Crispens MA et al. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst* 1998;90:447-454.
- Ishikawa F, Miyazone K, Hellman U et al. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature* 1989;338:557-562.
- Bussolino F, Di Renzo MF, Ziche M et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 1992;119:629-641.
- Grant DS, Kleinman HK, Goldberg ID et al. Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA* 1993;90:1937-1941.
- Heldin C-H. Structural and functional studies on platelet-derived growth factor. *EMBO J* 1992;11:4251-4259.
- Risau W, Drexler H, Mironov V et al. Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* 1992;7:261-266.
- Holmgren L, Glaser A, Pfeifer-Ohlsson S et al. Angiogenesis during extraembryonic development involves the spatiotemporal control of PDGF ligand and receptor gene expression. *Development* 1991;113:749-754.
- Moghaddam A, Zhang H-T, Fan T-PD et al. Thymidine phosphorylase is angiogenic and promotes tumor growth. *Proc Natl Acad Sci USA* 1995;92:998-1002.
- Kumar R, Yoneda J, Bucana CD et al. Regulation of distinct steps of angiogenesis by different angiogenic molecules. *Int J Oncol* 1998;12:749-757.
- Alon T, Hemo I, Itin A et al. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995;1:1024-1028.
- Benjamin LE, Kesher E. Conditioned switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci USA* 1997;94:8761-8766.
- Senger DR, Galli SJ, Dvorak AM et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983-985.
- Ferrara N, Houck KA, Jakeman LB et al. The vascular endothelial growth factor family of polypeptides. *J Cell Biochem* 1991;47:211-218.
- Yoshida A, Anand-Apte B, Zetter BR. Differential endothelial cell migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. *Growth Factors* 1996;13:57-64.
- Zetter BR. Cell motility in angiogenesis and tumor metastasis. *Cancer Invest* 1990;8:669-671.

33. Liotta LA, Thorgeirsson UP, Garbisa S. Role of collagenases in tumor cell invasion. *Cancer Metastasis Rev* 1982;1:277-288.
34. Singh RK, Reich R, Radinsky R et al. Expression of basic fibroblast growth factor is necessary but insufficient for production of metastasis. *Int J Oncol* 1997;10:23-31.
35. Pepper MS, Ferrara N, Orci L et al. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun* 1991;181:902-906.
36. Rosen EM, Grant DS, Kleinman HK et al. Scatter factor (hepatocyte growth factor) is a potent angiogenic factor in vivo. *Symp Soc Exp Biol* 1993;47:227-234.
37. Stoker M, Perryman M. An epithelial scatter factor released by embryo fibroblasts. *J Cell Sci* 1985;77:209-223.
38. Vernon RB, Sage H. Between molecules and morphology. Extracellular matrix and creation of vascular form. *Am J Pathol* 1995;147:873-883.
39. Collins PD, Connolly DT, Williams TJ. Characterization of the increase in vascular permeability induced by vascular permeability factor in vivo. *Br J Pharmacol* 1993;109:195-199.
40. Feng D, Nagy JA, Hipp J et al. Vesiculo-vacuolar organelles (VVOs) and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. *J Exp Med* 1996;183:1981-1986.
41. Roberts WG, Palade GE. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci* 1995;108:2369-2379.
42. Shweiki D, Itin A, Soffer D et al. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992;359:843-845.
43. Sunderkötter C, Steinbrink K, Goebeler M et al. Macrophages and angiogenesis. *J Leukocyte Biol* 1994;55:410-422.
44. Slavin J. The role of cytokines in wound healing. *J Pathol* 1996;178:5-10.
45. Polverini PJ, Leibovich JS. Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor-associated macrophages. *Lab Invest* 1984;51:635-642.
46. Gutman M, Singh RK, Yoon S et al. Leukocyte-induced angiogenesis and subcutaneous growth of B16 melanoma. *Cancer Biother* 1994;9:163-170.
47. Leek RD, Harris AL, Lewis CE. Cytokine networks in solid human tumors: regulation of angiogenesis. *J Leukocyte Biol* 1994;56:423-435.
48. Brown LF, Berse B, Jackman RW et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993;53:4727-4735.
49. Yoshiji H, Gomez DE, Shibuya M et al. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res* 1996;56:2013-2016.
50. Takahashi Y, Kitadai Y, Bucana CD et al. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964-3968.
51. Xerri L, Battyeni Z, Grob JJ et al. Expression of FGF1 and FGFR1 in human melanoma tissues. *Melanoma Res* 1996;6:223-230.
52. Takanami I, Tanaka F, Hashizume T et al. The basic fibroblast growth factor and its receptor in pulmonary adenocarcinomas: an investigation of their expression as prognostic markers. *Eur J Cancer* 1996;32A:1504-1509.
53. Engerman RL, Pfaffenbach D, Davis MD. Cell turnover of capillaries. *Lab Invest* 1967;17:738-743.
54. Hobson B, Denekamp J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *Br J Cancer* 1984;49:405-413.
55. Singh RK, Llansa N, Bucana CD et al. Cell density-dependent regulation of basic fibroblast growth factor expression in human renal cell carcinoma cells. *Cell Growth Differ* 1996;7:397-404.
56. Koura AN, Liu W, Kitadai Y et al. Regulation of vascular endothelial growth factor expression in human colon carcinoma cells by cell density. *Cancer Res* 1996;56:3891-3894.
57. Kitadai Y, Ellis LM, Takahashi Y et al. Multiparametric in situ messenger RNA hybridization analysis to detect metastasis-related genes in surgical specimens of human colon carcinomas. *Clin Cancer Res* 1995;1:1095-1102.
58. Swerlick RA, Lee KH, Li LJ et al. Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. *J Immunol* 1992;149:698-705.
59. Hitraya EG, Tan EM, Rudnicka L et al. Expression of extracellular matrix genes in adult human dermal microvascular endothelial cells and their regulation by heparin and endothelial cell mitogens. *Lab Invest* 1995;73:393-402.
60. Haraldsen A, Kvale D, Lien B et al. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. *J Immunol* 1996;156:2558-2565.
61. Auerbach R, Alby L, Morrissey LW et al. Expression of organ-specific antigens on capillary endothelial cells. *Microvasc Res* 1985;29:401-411.
62. Gumkowski F, Kaminska G, Kaminski M et al. Heterogeneity of mouse vascular endothelium. In vitro studies of lymphatic, large blood vessel and microvascular endothelial cells. *Blood Vessels* 1987;24:11-23.
63. McLean JW, Fox EA, Baluk P et al. Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. *Am J Physiol* 1997;273:H387-H404.
64. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;315:1650-1659.
65. Singh RK, Bucana CD, Gutman M et al. Organ site-dependent expression of basic fibroblast growth factor in human renal cell carcinoma cells. *Am J Pathol* 1994;145:365-374.
66. Singh RK, Llansa N, Bucana CD et al. Cell density-dependent regulation of basic fibroblast growth factor expression in human renal cell carcinoma cells. *Cell Growth Differ* 1996;7:397-404.
67. Kitadai Y, Ellis LM, Tucker SL et al. Multiparametric in situ mRNA hybridization analysis to predict disease recurrence in patients with colon carcinoma. *Am J Pathol* 1996;149:1541-1551.
68. Kitadai Y, Ellis LM, Takahashi Y et al. Multiparametric in situ mRNA hybridization analysis to detect metastasis-related genes in surgical specimens of human colon carcinoma. *Clin Cancer Res* 1995;1:1095-1102.
69. Herlyn M. Human melanoma: development and progression. *Cancer Metastasis Rev* 1990;9:101-112.
70. Singh RK, Bucana CD, Llansa N et al. Cell density-dependent modulation of basic fibroblast growth factor expression by human interferon- β . *Int J Oncol* 1996;8:649-656.
71. Gutman M, Singh RK, Xie K et al. Regulation of interleukin-8 expression in human melanoma cells by the organ environment. *Cancer Res* 1995;55:2470-2475.
72. Shweiki D, Itin A, Soffer D et al. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992;359:843-848.
73. Rak J, Filmus J, Finkenzeller G et al. Oncogenes as inducers of tumor angiogenesis. *Cancer Metastasis Rev* 1995;14:263-277.
74. Takahashi Y, Bucana CD, Liu W et al. Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: role of infiltrating cells. *J Natl Cancer Inst* 1996;88:1146-1151.
75. Takahashi Y, Mai M, Wilson MR et al. Site-dependent expression of vascular endothelial growth factor, angiogenesis and proliferation in human gastric carcinoma. *Int J Oncol* 1996;8:701-705.
76. Bocker EG, Rekenfeld C, Hamm H et al. Macrophages in melanocytic naevi. *Arch Dermatol Res* 1992;284:127-131.

77. Fidler IJ, Gersten DM, Kripke ML. Influence of immune status on the metastasis of three murine fibrosarcomas of different immunogenicities. *Cancer Res* 1979;39:3816-3821.
78. Sidky YA, Auerbach R. Lymphocyte-induced angiogenesis in tumor-bearing mice. *Science* 1976;192:1237-1238.
79. Koura AN, Liu W, Kitadai Y et al. Regulation of vascular endothelial growth factor expression in human colon carcinoma cells by cell density. *Cancer Res* 1996;56:3891-3894.
80. Noh R, Karp GI, Devereux DF. The effects of doxorubicin and mitoxantrone on wound healing. *Cancer Chemother Pharmacol* 1991;29:141-144.
81. Fidler IJ. Lymphocytes are not only immunocytes [editorial]. *Biomedicine* 1980;32:1-3.
82. Yoneda J, Killian JJ, Bucana CD et al. Angiogenesis and growth of murine colon carcinoma are dependent on infiltrating leukocytes. *Cancer Biother Radiopharm* 1999;14:221-230.
83. Meininger CJ, Zetter BR. Mast cells and angiogenesis. *Semin Cancer Biol* 1992;3:73-79.
84. Polverini PS, Cotran RS, Gimbrone MA Jr et al. Activated macrophages induce vascular proliferation. *Nature* 1977;269:804-805.
85. Sunderkötter C, Goebeler M, Schulze-Osthoff K et al. Macrophage-derived angiogenesis factors. *Pharmacol Ther* 1991;51:195-216.
86. Polverini PJ. How the extracellular matrix and macrophages contribute to angiogenesis-dependent diseases. *Eur J Cancer* 1996;32A:2430-2437.
87. Ingber D. Extracellular matrix and cell shape: potential control points for inhibition of angiogenesis. *J Cell Biochem* 1991;47:236-241.
88. Ingber DE, Folkman J. How does extracellular matrix control capillary morphogenesis? *Cell* 1989;58:803-805.
89. DiPietro LA, Polverini PJ. Angiogenic macrophages produce the angiogenic inhibitor thrombospondin 1. *Am J Pathol* 1993;143:678-684.
90. Lingen MW, Polverini PJ, Bouck N. Inhibition of squamous cell carcinoma angiogenesis by direct interaction of retinoic acid with endothelial cells. *Lab Invest* 1996;74:476-483.
91. Lingen MW, Polverini PJ, Bouck N. Retinoic acid induces cells cultured from oral squamous cell carcinomas to become anti-angiogenic. *Am J Pathol* 1996;149:247-258.
92. Reilly MS, Holmgren L, Shing Y et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994;79:315-328.
93. Dong Z, Kumar R, Yang X et al. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 1997;88:801-810.
94. Kumar R, Dong Z, Fidler IJ. Differential regulation of metalloelastase activity in murine peritoneal macrophages by granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor. *J Immunol* 1996;157:5104-5111.
95. Eckowitz RAB, Mulliken JB, Folkman J. Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med* 1992;326:1456-63.
96. White CW, Sondheimer HM, Crouch EC et al. Treatment of pulmonary hemangiomatosis with recombinant interferon- α -2a. *N Engl J Med* 1989;320:1197-1200.
97. Orchard PJ, Smith CM, Woods WG et al. Treatment of hemangioendotheliomas with alpha interferon. *Lancet* 1989;ii(8662):565-567.
98. Ricketts RR, Hailey RM, Corden BJ et al. Interferon- α -2a for the treatment of complex hemangiomas of infancy and childhood. *Ann Surg* 1994;219:605-614.
99. Ohlms LA, Jones DT, McGill TJ et al. Interferon- α -2a therapy for airway hemangiomas. *Ann Otol Rhinol Laryngol* 1994;103:1-8.
100. Bielenberg DR, Bucana CD, Sanchez R et al. Progressive growth of infantile cutaneous hemangiomas is directly correlated with hyperplasia and angiogenesis of adjacent epidermis and inversely correlated with expression of the endogenous angiogenesis inhibitor, IFN- β . *Int J Oncol* 1999;14:401-408.
101. Bielenberg DR, McCarty MF, Bucana CD et al. Expression of interferon- β is associated with growth arrest of murine and human epidermal cells. *J Invest Dermatol* 1999;112:802-809.
102. Bielenberg DR, Bucana CD, Sanchez R et al. Molecular regulation of UV-B-induced cutaneous angiogenesis. *J Invest Dermatol* 1998;111:864-872.
103. Uze G, Lutfalla G, Mogensen KE. α and β interferons and their receptor and their friends and relations. *J Interferon Cytokine Res* 1995;15:3-26.
104. Yaar M, Karassik RL, Schnipper LE et al. Effects of alpha and beta interferons on cultured human keratinocytes. *J Invest Dermatol* 1985;85:70-74.
105. Tamm I, Lin SL, Pfeffer LM et al. Interferons α and β as cellular regulatory molecules. *Interferon* 1987;9:13-74.
106. Rossi G. Interferons and cell differentiation. *Interferon* 1985;6:31-68.
107. Chatterjee D, Savarese TM. Posttranscriptional regulation of c-myc proto-oncogene expression and growth inhibition by recombinant human interferon- β ser17 in a human colon carcinoma cell line. *Cancer Chemother Pharmacol* 1992;30:12-20.
108. Resnitzky D, Yarden A, Zipori D et al. Autocrine β -related interferon controls c-myc suppression and growth arrest during hematopoietic cell differentiation. *Cell* 1986;46:31-40.
109. de Maeyer-Guignard J, de Maeyer E. Immunomodulation by interferons: recent developments. *Interferon* 1985;6:69-91.
110. Strander H. Interferon treatment of human neoplasia: effects on the immune system. *Adv Cancer Res* 1986;46:36-57.
111. Gresser I, Carnaud C, Maury C et al. Host humoral and cellular immune mechanisms in the continued suppression of Friend erythroleukemia metastases after interferon α/β treatment in mice. *J Exp Med* 1991;173:1193-1203.
112. Gresser I. Antitumor effects of interferon. *Acta Oncol* 1989;28:347-353.
113. Ferrantini M, Proietti E, Santodonato L et al. α 1-Interferon gene transfer into metastatic Friend leukemia cells abrogated tumorigenicity in immunocompetent mice: antitumor therapy by means of interferon-producing cells. *Cancer Res* 1993;53:1107-1112.
114. Gresser I, Belardelli F, Maury C et al. Injection of mice with antibody to interferon enhances the growth of transplantable murine tumors. *J Exp Med* 1983;158:2095-2107.
115. Gutterman JU. Cytokine therapeutics: lessons from interferon- α . *Proc Natl Acad Sci USA* 1994;91:1198-1205.
116. Gresser I. How does interferon inhibit tumor growth? *Interferon* 1985;6:93-126.
117. Fleischmann WR, Fleischmann CM. Mechanisms of interferons antitumor actions. In: Baron S, Coppenhaver DH, Dianzani F et al, eds. *Interferon: Principles and Medical Applications*. Galveston, TX: University of Texas Press, 1992:299-309.
118. Goriach A, Herter P, Hentschel H et al. Effects of rIFN- β and rIFN- γ on growth and morphology of two human melanoma cell lines: comparison between two- and three-dimensional culture. *Int J Cancer* 1994;56:249-254.
119. Johns TG, Mackay IR, Callister KA et al. Antiproliferative potencies of interferons on melanoma cell lines and xenografts: higher efficacy of interferon-beta. *J Natl Cancer Inst* 1992;84:1185-1190.
120. Sica G, Fabbri L, Castagnetta L et al. Antiproliferative effect of interferons on human prostate carcinoma cell lines. *Urol Res* 1989;17:111-115.
121. Heyns AP, Eldor A, Vlodavsky I et al. The antiproliferative effect of interferon and the mitogenic activity of growth factors

- are independent cell cycle events. Studies with vascular smooth muscle cells and endothelial cells. *Exp Cell Res* 1985;161:297-306.
122. Friesel R, Komoriya A, Maciag T. Inhibition of endothelial cell proliferation by gamma-interferon. *J Cell Biol* 1987;104:689-696.
 123. Ruszczak Z, Detmar M, Imcke E et al. Effects of rIFN- α , - β , and - γ on the morphology, proliferation, and cell surface antigen expression of human dermal microvascular endothelial cells in vitro. *J Invest Dermatol* 1990;95:693-699.
 124. Hicks C, Breit SN, Penny R. Response of microvascular endothelial cells to biological response modifiers. *Immunol Cell Biol* 1989;67(5):271-277.
 125. Groopman JE, Gottlieb MS, Goodman J et al. Recombinant alpha-2 interferon therapy for Kaposi's sarcoma associated with the acquired immunodeficiency syndrome. *Ann Intern Med* 1984;100:671-676.
 126. Real FX, Oertgen HF, Krown SE. Kaposi's sarcoma and the acquired immunodeficiency syndrome: treatment with high and low doses of recombinant leukocyte A interferon. *J Clin Oncol* 1986;4:544-551.
 127. Rios A, Mansell PW, Newell GR et al. Treatment of acquired immunodeficiency syndrome-related Kaposi's sarcoma with lymphoblastoid interferon. *J Clin Oncol* 1985;3:506-512.
 128. Mitsuyasu RT. Interferon alpha in the treatment of AIDS-related Kaposi's sarcoma. *Br J Haematol* 1991;79[suppl 1]:69-73.
 129. Legha SS. The role of interferon alfa in the treatment of metastatic melanoma. *Semin Oncol* 1997;24:S24-31.
 130. Tucker SB. Interferon-alpha treatment of basal cell and squamous cell skin tumors. *Cancer Bull* 1993;45:270-274.
 131. Stadler WM, Kuzel TM, Raghavan D et al. Metastatic bladder cancer: advances in treatment. *Eur J Cancer* 1997;33[suppl]:S23-26.
 132. Nanus DM, Schmitz-Drager BJ, Motzer RJ et al. Expression of basic fibroblast growth factor in primary human renal tumors: correlation with poor survival. *J Natl Cancer Inst* 1994;85:1597-1599.
 133. Nguyen M, Watanabe H, Budson AE et al. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. *J Natl Cancer Inst* 1994;86:356-361.
 134. O'Brien TS, Smith K, Cranston D et al. Urinary basic fibroblast growth factor in patients with bladder cancer and benign prostatic hypertrophy. *Br J Urol* 1995;76:311-314.
 135. Singh RK, Gutman M, Bucana CD et al. Interferons alpha and beta downregulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci USA* 1995;92:4562-4566.
 136. Dinney CPN, Bielenberg DR, Reich R et al. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 1998;58:808-814.
 137. Broudy-Boye D, Zetter BR. Inhibition of cell motility by interferon. *Science* 1980;208:516-518.
 138. Fukuzawa K, Horikoshi T. Inhibitory effect of human fibroblast interferon (HuIFN- β) on the growth and invasive potential of cultured human melanoma cells in vitro. *Br J Dermatol* 1992;126:324-330.
 139. Stout AJ, Gresser I, Thompson WD. Inhibition of wound healing in mice by local interferon α/β injection. *Int J Exp Pathol* 1993;74:79-85.
 140. Dvorak HF, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. *J Natl Cancer Inst* 1989;81:497-502.
 141. Sidky YA, Borden EC. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. *Cancer Res* 1987;47:5155-5161.
 142. Singh RK, Gutman M, Llansa N et al. Interferon- β prevents the upregulation of interleukin-8 expression in human melanoma cells. *J Interferon Cytokine Res* 1996;16:577-584.
 143. Fabra A, Nakajima M, Bucana CD et al. Modulation of the invasive phenotype of human colon carcinoma cells by organ-specific fibroblasts of nude mice. *Differentiation* 1992;52:101-110.
 144. Gohji K, Nakajima M, Fabra A et al. Regulation of gelatinase production in metastatic renal cell carcinoma by organ-specific fibroblasts. *Jpn J Cancer Res* 1994;85:152-160.
 145. Bielenberg DR, Fidler IJ, Bucana CD. Constitutive expression of interferon-beta in differentiated epithelial cells exposed to environmental stimuli. *Cancer Biother Radiopharm* 1998;13:375-382.
 146. Bielenberg DR, McCarty ME, Bucana CD et al. Expression of interferon- β is associated with growth arrest of murine and human epidermal cells. *J Invest Dermatol* 1999;112:802-809.
 147. Xu L, Xie K, Fidler IJ. Therapy of human ovarian cancer by transfection with the murine interferon beta gene: role of macrophage-inducible nitric oxide synthase. *Hum Gene Ther* 1998;9:2699-2708.
 148. Xie K, Bielenberg D, Huang S et al. Abrogation of tumorigenicity and metastasis of murine and human tumor cells by transfection with the murine IFN-beta gene: possible role of nitric oxide. *Clin Cancer Res* 1997;3:2283-2294.
 149. Dong Z, Greene G, Pettaway C et al. Suppression of angiogenesis, tumorigenicity, and metastasis by human prostate cancer cells engineered to produce interferon- β . *Cancer Res* 1999;59:872-879.

Tu
to

Philip

V
t
ture
indir
withi
plus
or ox
eral
rectly
peut
perc
capi
mor
fect
end
the
gen
mo
che
to cang
cul
no
p
va
alc
ce
ve
ag
oc
tu
g
lca
v
I
t
-
t
t

Bevacizumab

Bevacizumab (Avastin; Genentech/Roche), an antibody against vascular endothelial growth factor, was approved by the US FDA in February 2004 for the first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy. It is the first approved agent to target tumour angiogenesis.

Angiogenesis — the development of new blood vessels from pre-existing vasculature — has a key role in normal development, but also in several diseases, such as cancer. For a tumour to grow beyond a certain size, it needs a network of blood vessels that supply nutrients and oxygen, and remove waste products¹. So, understanding how angiogenesis is regulated in cancer has been a major area of cancer research, particularly in the past decade.

Basis of discovery

The hypothesis that inhibiting angiogenesis might be an effective anticancer strategy was put forward more than 30 years ago². Earlier experiments had provided evidence that

tumour angiogenesis was mediated by diffusible factors produced by tumour cells, and this hypothesis stimulated efforts to identify these factors¹.

In the 1980s, such efforts led to the isolation of vascular endothelial growth factor (VEGF), a potent stimulator of the growth of endothelial cells, the main type of cell in the inside lining of blood vessels¹. It is now known that VEGF, which activates receptor tyrosine kinases on the surface of endothelial cells (FIG. 1), is a key regulator of normal and pathological blood vessel growth¹. In the early 1990s, the demonstration that inhibition of VEGF-induced angiogenesis using a monoclonal antibody against VEGF markedly suppressed tumour growth *in vivo*³ led to the development of bevacizumab.

Drug properties

Bevacizumab is a humanized version of the monoclonal antibody against VEGF that was used in early proof-of-principle experiments^{3,4}. It binds to VEGF, which prevents its interaction with the VEGF receptor tyrosine kinases

VEGFR1 and VEGFR2 (FIG. 1), and inhibits the growth of human tumour cell lines in mice¹. Following early clinical trials showing that bevacizumab as a single agent was relatively non-toxic, and that it could be added to standard cytotoxic chemotherapy regimes, large clinical trials were initiated in several cancer types, including colorectal cancer¹.

Clinical data

It has been estimated that colorectal cancer is the fourth largest cause of cancer deaths⁵. For many years, chemotherapy of the disease has been based on traditional cytotoxic drugs, in particular 5-fluorouracil (5-FU) in combination with leucovorin (LV). More recently, the addition of the cytotoxic drugs irinotecan (Camptosar; Pfizer) or oxaliplatin (Eloxatin; Sanofi-Aventis) to 5-FU/LV regimes has been shown to prolong survival in advanced colorectal cancer.

Bevacizumab, in combination with 5-FU-based chemotherapy, was studied in randomized, controlled trials as a first-line treatment for patients with metastatic carcinoma of the colon or rectum^{6,7}. In the pivotal Phase III study, which involved more than 800 patients, bevacizumab (5 mg per kg every 2 weeks) or placebo was given to the patients in addition to bolus-IFL (irinotecan 125 mg per m² intravenously, 5-FU 500 mg per m² intravenously and LV 20 mg per m² intravenously once weekly for 4 weeks every 6 weeks). Median overall survival was significantly increased from 15.6 months in the bolus-IFL + placebo arm to 20.3 months in the bolus-IFL + bevacizumab arm^{6,7}. Similar increases were also seen in progression-free survival (6.4 versus 10.6 months), overall response rate (35 % versus 45%) and duration of response (7.1 months versus 10.4 months).

Bevacizumab was generally well tolerated in these clinical trials; however, some serious and unusual toxicities were noted. In particular, bevacizumab was associated with gastrointestinal perforations and wound healing complications in ~2% of patients⁷. Other adverse events associated with bevacizumab use include thromboembolic complications, hypertension, bleeding and proteinuria⁷.

Indications

Bevacizumab, used in combination with intravenous 5-FU-based chemotherapy, is approved by the FDA for the first-line treatment of patients with metastatic carcinoma of the colon or rectum⁷.

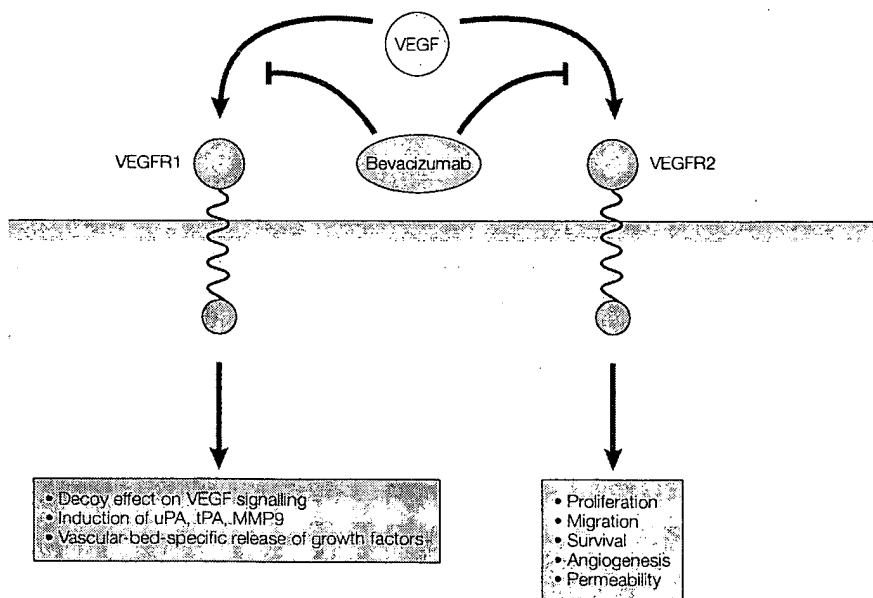
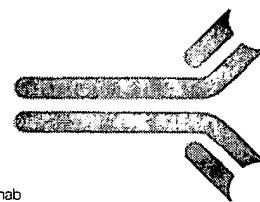


Figure 1 | Simplified view of VEGF signalling and tumour angiogenesis. The receptors for vascular endothelial growth factor (VEGF, also known as VEGF-A) — VEGFR1 (also known as Flt1) and VEGFR2 (also known as Flk1 or KDR) — are expressed on the surface of blood endothelial cells. VEGFR2 is thought to be the major mediator of endothelial cell mitogenesis, survival and microvascular permeability, whereas VEGFR1 does not seem to mediate an effective mitogenic signal in endothelial cells, but does have other activities that can be important in tumour growth and metastasis, including the induction of matrix metalloproteinases (MMPs). tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator. Adapted from REF. 1.



Bevacizumab

ANTI-VEGF THERAPIES | VIEW FROM THE CLINIC

Analysing clinical issues for anti-VEGF therapies is Lee M. Ellis, M.D., Professor of Surgical Oncology and Professor of Cancer Biology at the University of Texas MD Anderson Cancer Center, Texas, USA. His research interests are defining the role of angiogenesis in primary and metastatic human colorectal, pancreatic and gastric cancers, and evaluating mechanisms of anti-angiogenic therapies, such as those targeted against VEGF.

What impact have anti-VEGF therapies such as bevacizumab had on the treatment of cancer?

The results from the original Phase III trials comparing IFL to IFL and bevacizumab⁶, and the subsequent FDA approval of bevacizumab in combination with intravenous 5-FU-based regimens, have changed the standard of care for patients with metastatic colorectal cancer (CRC). More recently, it has been announced that in Phase III trials, bevacizumab provided benefit to patients with advanced non-small-cell lung cancer (NSCLC). In addition, Phase I/II studies have suggested that the addition of bevacizumab to other therapeutic modalities (chemotherapy and radiation therapy) might be efficacious in patients with other malignancies, such as pancreatic carcinoma, renal-cell carcinoma and locally advanced rectal cancer.

Another Phase III trial in metastatic CRC evaluated patients randomized to FOLFOX with or without the VEGF receptor tyrosine kinase inhibitor PTK787/ZD22584. It has been announced that after ~1,200 patients had been accrued, an investigator analysis demonstrated an improvement in progression-free survival, but a central radiology review did not note a statistically significant improvement in progression-free survival. Data on overall survival should be available in 2006.

How well are the mechanisms of benefit of anti-VEGF therapies understood?

It is important to differentiate anti-VEGF therapy from other anti-angiogenic therapies, many of which are specifically intended to disrupt endothelial cell signalling or survival pathways. VEGF is a pluripotent factor that has multiple effects on the vasculature, including induction of angiogenesis and enhancement of endothelial cell survival, the ability to induce vascular permeability, and production of a dilated and disorganized vascular network. This altered tumour vascular network leads to inefficient blood flow, which can theoretically be 'normalized' with anti-VEGF therapy, augmenting delivery of chemotherapy and oxygen⁸.

It was initially believed that the VEGF receptors (VEGFRs) are present only on endothelial cells, but recent studies have demonstrated that VEGFRs are present on tumour cells^{9,10}. So, another potential mechanism of action of anti-VEGF therapy is a direct effect on tumour cells, where it might inhibit processes involved in tumour progression and metastasis.

In addition to the above novel mechanisms for anti-VEGF therapy, it is still believed that anti-VEGF therapy can inhibit tumour angiogenesis. A detailed analysis has shown that the addition of bevacizumab to chemotherapy leads to a remarkable improvement in progression-free survival relative to the incremental improvement in response rate as seen with chemotherapy alone (A. Grothey, personal communication). This observation suggests that anti-VEGF therapy might indeed be anti-angiogenic, although this is indirect evidence.

How do you see anti-VEGF therapy evolving in the next few years?

Although most studies with bevacizumab have been carried out in patients with advanced-stage disease, future studies will include its use in combination with other therapies in the neoadjuvant and adjuvant settings. Interesting results have already been obtained with the use of bevacizumab in addition to chemoradiation therapy for locally advanced rectal and pancreatic cancers^{11,12}, and ongoing trials in CRC should provide some insight into the appropriate use of this agent in the adjuvant setting. However, one must consider the long-term effects of bevacizumab. In a meta-analysis of randomized Phase III trials, a 2.3-fold increase in arterial thrombotic events, including stroke, myocardial infarction, angina and transient ischaemic attacks, was noted. This is particularly important in patients who will be receiving bevacizumab in the adjuvant setting, and long-term follow up of cardiovascular events is crucial in such studies, especially as many patients would remain disease-free without therapy or with chemotherapy alone as adjuvant therapy.

Although anti-angiogenic therapy was originally intended for use as single-agent therapy, and was then combined with chemotherapy, there is also great potential for use in combination with other biological agents. One very interesting observation during the past year has been the results from the BOND2 study (a follow-up of the BOND1 study of cetuximab with or without irinotecan in irinotecan-refractory patients). In this study, patients with metastatic CRC who had progressed on irinotecan-based therapy were randomized to receive cetuximab

plus bevacizumab or cetuximab plus bevacizumab plus irinotecan¹³. The combination of the two biological agents alone led to a remarkable 23% response rate in the refractory setting, and the addition of irinotecan to the two biological agents led to a 38% response rate. These results suggest that biological agents can be combined without undue toxicity and that dual targeting of various mediators of tumour progression is efficacious in and of itself. However, the addition of chemotherapy to the biological agents is likely to achieve an even better result.

In summary, it is clear that bevacizumab improves the effects of chemotherapy in patients with metastatic CRC and NSCLC, and possibly other cancers. The efficacy of single-agent anti-VEGF therapy requires further study, in both the advanced-disease setting as well as the adjuvant setting. It is imperative that we determine the underlying mechanism of action of anti-VEGF drugs in order to develop predictive markers and better define the optimal use of these agents, as well as their potential short- and long-term toxicities.

Lee M. Ellis is at the University of Texas MD Anderson Cancer Center, PO Box 301402, Houston, Texas 77230-1402, USA. Peter Kirkpatrick is at Nature Reviews Drug Discovery. Correspondence to L.M.E. & P.K. e-mails: lellis@mdanderson.org; p.kirkpatrick@nature.com

doi:10.1038/nrd1727

1. Ferrara, N. *et al.* Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature Rev. Drug Discov.* **3**, 391–400 (2004).
2. Folkman, J. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **285**, 1182–1186 (1971).
3. Kim, K. J. *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362**, 841–844 (1993).
4. Presta, L. G. *et al.* Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.* **57**, 4593–4599 (1997).
5. Parkin, D. M. *et al.* Cancer burden in the year 2000. The global picture. *Eur. J. Cancer* **37**, S4–S66 (2001).
6. Hurwitz, H. *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* **350**, 2335–2342 (2004).
7. FDA labelling information [online] <<http://www.fda.gov/cder/foi/label/2004/125085bl.pdf>> (2004).
8. Jain, R. K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62 (2005).
9. Dias, S. *et al.* Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *J. Clin. Invest.* **106**, 511–521 (2000).
10. Fan, F. *et al.* Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. *Oncogene* **24**, 2647–2653 (2005).
11. Willeit, C. G. *et al.* Direct evidence that the VEGF-specific antibody bevacizumab has antitumor effects in human rectal cancer. *Nature Med.* **10**, 145–147 (2004).
12. Crane, C. H. *et al.* Preliminary results of a Phase I study of rhuMab VEGF (bevacizumab) with concurrent radiotherapy (XRT) and capecitabine (CAP). *Proc. ASCO Gastrointestinal Cancers Symp.* **A85** (2004).
13. Saltz, L. B. *et al.* Interim report of randomized Phase II trial of cetuximab/bevacizumab/irinotecan (CBi) versus cetuximab/bevacizumab (CB) in irinotecan-refractory colorectal cancer. *Proc. ASCO Gastrointestinal Cancers Symp.* **A169b** (2005).

Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis

Walter Kolch¹, Georg Martiny-Baron², Arnd Kieser¹, and Dieter Marmé²

¹ *Hämatologikum der GSF, Institut für Klinische Molekularbiologie und Tumorgenetik, Marchioninistr. 25, D-81377 München, Germany;* ² *Klinik für Tumorbologie, Institut für Molekulare Medizin und Naturstoffforschung, Breisacherstr. 117, D-79106 Freiburg, Germany*

Key words: vascular endothelial growth factor (VEGF), VEGF receptors, vascular permeability factor, regulation

Summary

Vascular endothelial growth factor (VEGF) / vascular permeability factor (VPS) plays a crucial role for the vascularization of tumors including breast cancers. Tumors produce ample amounts of VEGF, which stimulates the proliferation and migration of endothelial cells (ECs), thereby inducing tumor vascularization by a paracrine mechanism. VEGF receptors (VEGF-Rs) are highly expressed by the ECs in tumor blood vessels. VEGF expression can be induced in various cell types by a number of stimuli including hypoxia, differentiation, growth factors and tumor promoters of the phorbol ester class, such as TPA. The VEGF inductive pathways comprise kinases, oncogenes, tumor suppressor genes, and steroid hormone transcription factors, many of which seem to converge on the activator protein (AP-1) transcription factor. Much less is known about the regulation of VEGF-R expression, which is restricted to ECs. This expression is greatly enhanced in diseased tissue such as solid tumors. So far, it appears that growth factors, cytokines, and tumor promoters are involved in the control of VEGF-R expression. Here we review current knowledge about the regulation of the expression of VEGF and its receptors.

Introduction

The seminal findings of Folkman and colleagues that tumors require proper vascularization for growth [1,2] have sparked an intense interest in growth factors which promote angiogenesis. Especially in the case of breast cancer, the assessment of the extent of tumor vascularization in the form of blood vessel counts is beginning to emerge as a novel prognostic indicator which is predictive of both relapse-free and overall survival [3-6].

The sprouting of new blood vessels is initiated

by the degradation of the extracellular matrix, and the migration and concomitant proliferation of ECs. A large variety of growth factors have been described which might participate in this complex process (for instance reviewed in [7,8]). A steadily growing number of reports, however, have pinpointed VEGF, also known as VPS, as the pivotal *in vivo* mediator of physiological and pathophysiological angiogenesis (reviewed in [9]). VEGF is encoded by a single gene, which gives rise to at least four protein products, VEGF₂₀₆, 189, 165, 121 (named by the number of amino acids), which are generated by alternative splicing

of the mRNA [10-12]. VEGF can elicit many of the pleiotropic processes associated with angiogenesis. It is both a mitogen and a potent chemoattractant for ECs *in vitro* and *in vivo* [13-17]. In addition, VEGF induces ECs to release other factors which are involved in tissue remodelling and blood coagulation (reviewed in [18]). By enhancing vascular permeability, VEGF/VPS promotes the deposition of fibrin in the surrounding tissue, which favors the formation of a vascularized tumor stroma by fostering tissue infiltration with monocytes, fibroblasts, and ECs [18-20]. Intriguingly, monocytes have been reported to respond to VEGF/VPS with migration and activation [21] including the production of VEGF [22-24], which may sustain a positive feedback circuitry that further promotes neovascularization in situations of tumor growth or chronic inflammation.

Many different cell types are able to produce VEGF, but its mitogenic activity seems to be confined to ECs [14-17], which express both high and low affinity binding sites for VEGF [25-27]. Recently, three structurally related PDGF receptor-like tyrosine kinases, flt-1 [28], flt-4 [29], and flk-1/kdr [30], have been identified as putative VEGF receptors. Flt-1 and flk-1/kdr bind VEGF with high affinity, appear to be exclusively expressed on ECs, and have been shown to mediate at least part of the cellular responses to VEGF [28,30,31]. Flt-4 does not bind VEGF, but rather seems to interact with another yet unidentified ligand [32].

The involvement of VEGF in tumor neoangiogenesis was first indicated by observations that many tumor cell lines produce VEGF [33] and by the abundance of VEGF in tumors [34-36]. Moreover, VEGF receptor expression is upregulated in ECs of tumor blood vessels [37,38]. The VEGF concentration is highest in the vicinity of blood vessels, and antibodies to VEGF stain the ECs. Immune rejection of experimental tumors is accompanied by a rapid loss of the VEGF stain of blood vessels [39]. However, as evidenced by *in situ* hybridization with VEGF specific probes, the ECs do not produce VEGF by themselves, but

are stimulated in a strictly paracrine fashion [34,37,38]. The rich source of VEGF is provided by tumor infiltrating macrophages and mast cells as well as by the tumor cells themselves (reviewed in [18,40]). The few studies done with primary human tumor material suggest that the expression levels of VEGF in brain [35,41,42], bladder [43], kidney [43,44], ovarian (D.M., unpublished data), and gastrointestinal tract tumors [45] increase with progression towards higher malignancy and seem to correlate with the extent of tumor vascularization. In primary breast carcinomas, VEGF expression is associated with high microvessel density and poor prognosis due to early relapse [46]. Furthermore, tumor blood vessels generally exhibit increased permeability, which in some situations, e.g. in brain tumors, causes severe clinical symptoms due to compression of the surrounding tissue [42,47].

The importance of VEGF as a tumor neoangiogenesis factor was directly substantiated by showing that VEGF expression is a prerequisite for tumor growth. Exogenous overexpression of VEGF in chinese hamster ovary (CHO) cells fails to transform the cells by *in vitro* criteria, but nevertheless bestows them with the ability to form small, well vascularized tumors in mice [48]. Likewise, transfection with a VEGF expression plasmid increases the tumorigenicity of HeLa cells as well as tumor vascularization [49]. Injection of VEGF neutralizing antibodies inhibits the growth of transplanted tumors in mice without affecting the proliferation of the tumor cells *in vitro* [49,50]. Similarly, interfering with VEGF receptor function by expression of a dominant negative mutant of the flk-1 VEGF receptor profoundly reduces tumor growth in mice [51].

Regulation of VEGF expression

Research in this area is receiving growing attention, since understanding the molecular mechanisms harbors the prospect for the rational design of antiangiogenic therapies by interfering

with
ience
to th
the
invol

Diffe

The e
sugg
the v
VEG
the d
embr
physi
mensi
the e
down
maint
[52,5
stimu
and it

Bc
strong
(PGE)
the ex
sumat
induct
dexam
acid,
osteob
VEGF
promo
the sp
blast c
[58].

Ad
format
adipoc
factors
[59,60
pads v
inducti
the adi
express
observ

with VEGF expression. For the reader's convenience this paragraph will be subdivided according to the VEGF-inducing stimuli and conclude with the intracellular signalling pathways that are involved in the regulation of VEGF expression.

Differentiation

The expression pattern of VEGF and its receptors suggests that VEGF governs the development of the vascular system of the embryo [31,52,53]. VEGF and its receptors are highly expressed in the developing vascular system of mammalian embryos. In the adult, where neovascularization physiologically occurs only in connection with the menstrual cycle in the ovary and endometrium, the expression of VEGF and its receptors is downregulated, and VEGF probably serves to maintain the viability and proper function of ECs [52,54-56]. Nothing is known about the inductive stimuli which regulate the expression of VEGF and its receptors during development.

Bone formation requires angiogenesis and is strongly stimulated by prostaglandins E1 and E2 (PGE). PGE treatment of osteoblasts increases the expression of VEGF mRNA and protein, presumably on the transcriptional level. VEGF induction is blocked by cAMP antagonists and dexamethasone, but can be enhanced by retinoic acid, which induces differentiation of pre-osteoblasts [57]. Another report shows that VEGF raises cAMP levels in osteoblasts and promotes migration and differentiation, leading to the speculation that VEGF contributes to osteoblast differentiation by an autocrine mechanism [58].

Adipocyte differentiation coincides with the formation of new blood vessels. Cultured 3T3 adipocytes have been shown to secrete angiogenic factors in a differentiation dependent fashion [59,60] and to give rise to well vascularized fat pads when injected into mice [61]. *In vitro* induction of the differentiation of 3T3 cells along the adipocyte lineage is accompanied by enhanced expression of VEGF mRNA and protein. Similar observations were made in the case of myogenic

differentiation induced in C2C12 myoblast cells [62]. The underlying mechanisms for the induction of VEGF expression are not resolved yet, but might involve protein kinase C (PKC) and cAMP dependent protein kinase (PKA) mediated signalling pathways, since both the PKC activator TPA and cAMP analogues could recapitulate VEGF induction in 3T3 preadipocytes as well as differentiated adipocytes. The same study showed that transformed PC12 cells express VEGF. Differentiation of PC12 into non-malignant neuronal cells leads to a marked decline in VEGF expression. It is certainly premature to draw definite conclusions from these studies, since the role of VEGF for differentiation per se is not known, nor have the *in vivo* effects of VEGF been investigated in this context. Nevertheless, the results so far tempt one to speculate that differentiating cells regulate their blood supply by modulating VEGF expression. Interestingly, both differentiation processes which physiologically elicit vascularization (e.g. adipocyte differentiation), as well as malignant dedifferentiation (exemplified by PC12 cells), seem to utilize the modulation of VEGF expression for promoting angiogenesis.

Hypoxia

Hypoxia increases vascular permeability [63,64] and provides a potent stimulus for neovascularization [65]. In glioblastomas, VEGF is highly expressed in the immediate vicinity of necrotic lesions, which presumably are caused by hypoxemia [37,66]. Oxygen deprivation strongly induces VEGF expression in established [66-68] and primary [69] cell lines in culture. The mechanism of induction is poorly understood, but resembles the regulation of erythropoietin expression by hypoxemia [67]. Erythropoietin expression is regulated at the transcriptional level as well as postranscriptionally. Both genes seem to respond to a common sensor of oxygen tension in the cell — probably a heme protein, as inferred from the susceptibility to stimulation by cobalt chloride and inhibition by carbon monoxide. In both cases the hypoxic induction of mRNA ex-

pression is dependent on protein synthesis, but once induced, inhibition of protein synthesis prolongs the half life of the mRNAs [67]. The promoter of the VEGF gene contains two sequence motifs which share high homology with the hypoxia-responsive enhancer element of the human erythropoietin gene. A further stretch of sequence homology is found in the 3'-untranslated regions of erythropoietin and VEGF mRNAs [67]. Hypoxia has been shown to increase the binding of a protein factor to this site in the erythropoietin mRNA [70], which serves as a transcriptional enhancer [71].

A hint about the signalling pathways involved comes from the observation that hypoxia and cobalt chloride also induce the expression of *c-fos* and *jun* family genes [67,72,73]. Jun family homodimers or Fos/Jun heterodimers constitute the AP-1 transcription factor (reviewed in [74]), which might play a prominent role in the regulation of VEGF expression (also see below). The VEGF promoter features four AP-1 sites [10], and its transcription can be stimulated by coexpression of *v-fos* or *c-jun* in transient transfection assays (A. Technau, A.K., W.K., and D.M., unpublished data). Compared with mitogens, the induction of *fos* and *jun* mRNAs by hypoxia or cobalt occurs with strikingly delayed kinetics, which, however, coincide with the time course of VEGF mRNA expression. Little is known about upstream signal transducers activated by hypoxia, but work with chemical inhibitors suggests that kinases different from PKC are involved [75,76]. Plausible candidates might be found amongst the recently discovered family of stress-activated protein kinases (SAPK) / *c-jun* NH₂-terminal kinases (JNK). In contrast to PKC they are activated by various conditions of cellular stress, but not by mitogenic stimuli. They can directly activate AP-1 transcriptional function by phosphorylating the Jun transactivation domain (reviewed in [77]).

Steroid hormones

As anticipated for a physiological mediator of angiogenesis, the expression of VEGF mRNA and

protein is increased in organs actively undergoing vascularization. Estrogen-induced regeneration of the uterine endometrium after menses includes the proliferation and an increase in permeability of the spiral uterine arteries [78,79]. Estrogen rapidly stimulates the expression of VEGF mRNA in the rat uterus in a manner not requiring prior protein synthesis [80]. *In vitro* studies with endometrial carcinoma cells confirm the induction of VEGF by estrogen [81]. A more detailed spatial resolution by *in situ* hybridization shows that VEGF is expressed in different steroid responsive and steroidogenic cell types in the mouse [81]. In mouse ovaries VEGF induction ensues in a coordinated spatial and temporal fashion restricting expression to areas which acquire new capillary beds during the female cycle, such as the maturing and ovulating follicle and the developing corpus luteum [81,82]. After ovulation the avascular granulosa cell layer of the empty follicle is rapidly vascularized. Granulosa cells express VEGF [81,83], and in primary cultures of bovine ovarian granulosa cells the transcription of the VEGF gene can be induced by both estrogen and luteinizing hormone (LH) [83]. LH elevates cAMP levels in the cell by stimulating adenylate cyclase. The stimulation of VEGF expression by LH can consequently be mimicked by forskolin, a drug which directly activates adenylate cyclase. This type of induction occurs despite inhibition of protein synthesis and may be mediated by the cAMP responsive activator protein 2 (AP-2) consensus motifs [84] in the promoter of the VEGF gene.

The VEGF promoter lacks steroid responsive elements, and induction of the VEGF gene by estrogen therefore must occur indirectly. Several possibilities can be envisioned. Estrogen raises cAMP levels in cultured breast cancer and uterine cells, which suffice to activate transcription of cAMP reporter genes [85] and thus might induce the VEGF gene via AP-2 transcription factors. In hormone responsive cells, estrogen also induces the transcription of the *c-jun* and *c-fos* genes [86], and hence the formation of AP-1 transcription factor complexes, which eventually could activate

VEGF transcription. Both estrogen and progesterone have been reported to augment the production of phosphoinositides and phosphatidylcholine in breast cancer cells [87,88]. These lipids are the precursors for a variety of second messenger signalling molecules including the physiological PKC activator diacylglycerol [89]. The biological significance of this observation is unclear, but it is plausible to assume that increases in the phospholipid pool facilitate PKC activation and PKC dependent VEGF induction. Finally, estrogen acutely triggers tyrosine phosphorylation in MCF-7 breast cancer cells, presumably by activating the c-Src tyrosine kinase. This stimulation is mediated by the estrogen receptor (ER), since the induction of tyrosine phosphorylation can be inhibited by antiestrogens and does not take place in ER-negative COS cells [90]. Interestingly, Src not only stimulates cell proliferation, but can also induce VEGF expression (see below). It remains to be tested, however, whether in breast carcinomas VEGF expression is related to the ER status. Blood vessel counts do not appear to be significantly linked to the expression of ERs [3]. *Prima vista*, this seems to discount estrogen as a potential regulator of VEGF mediated angiogenesis. The multiple ways by which estrogen could induce VEGF expression, on the other hand, indicate that the specific genetic status of the tumor cell might determine whether estrogen can stimulate VEGF expression. For instance, it will be instructive to look for a correlation between ER status, mutations in the p53 tumor suppressor gene, and VEGF expression. As discussed below, wildtype p53 constrains the expression of the VEGF gene, and subversion of p53 function might be required for VEGF induction by estrogen.

Peptide growth factors

Several peptide growth factors including transforming growth factors TGF α [91] and β [92], epidermal growth factor (EGF) [93], and platelet-derived growth factor (PDGF) [68,94], have been

reported to augment VEGF expression in a variety of non-endothelial cell types in culture. All of these factors possess a wide range of diverse biological actions including inherent angiogenic properties [7,8]. This precludes firmly establishing a role of these growth factors in VEGF induction in tumors at the moment. What is striking, however, is that the set of known VEGF-inducing growth factors is virtually identical to the panel of growth factors implicated in breast and other cancers.

Although TGF β strongly stimulates angiogenesis *in vivo* [95], it represses the proliferation of ECs *in vitro* [95-97]. TGF β induces VEGF expression in vascular smooth muscle cells [68], fibroblasts, and epithelial cells [92], but not in ECs, suggesting that it exerts its angiogenic effects through a paracrine mechanism involving VEGF. The role of TGF β in breast cancer is controversial. High expression of TGF β_1 has been linked to both high [98] and low [99] metastatic potential. These discrepancies might be due to different detection methods, or else might reflect the complex biological effects of TGF β . Besides VEGF, TGF β can induce PDGF and basic fibroblast growth factor (bFGF) [92], whose effects on ECs synergize with VEGF [100]. TGF β might also enhance the growth of breast carcinomas by suppression of natural killer cell activity [101]. On the other hand, TGF β can inhibit the proliferation of many epithelial cell lines including breast cancer cells [102]. Thus, the biological outcome appears to depend on the balance between the different processes triggered by TGF β .

TGF α or EGF induce VEGF expression in cultured epidermal keratinocytes [91] and glioblastoma cells [93], respectively. Both growth factors bind to and signal through the EGF receptor (EGF-R), which is highly expressed by these cell types, and whose overexpression is commonly found in glioblastomas (for recent review see [103]). Psoriatic skin lesions as well as glioblastomas present with vascular proliferation and hyperpermeability, which may be attributed to the TGF α /EGF-triggered production of

VEGF. High expression of TGF α , EGF, and EGF-Rs is also found in an appreciable fraction especially of advanced breast cancers, although the prognostic value of these parameters is under dispute (recently reviewed in [104-106]). By virtue of the ability to induce tyrosine phosphorylation and Ras activation (reviewed in [107]), the EGF-R is expected to be able to stimulate not only proliferation but VEGF expression as well.

PDGF induces VEGF mRNA in human vascular smooth muscle cells [68] and in murine NIH 3T3 fibroblasts [94]. This induction appears to be mediated by PKC. Overexpression of PKC α enhances VEGF expression, whereas pharmacological depletion of PKC blocks induction. High PDGF levels in plasma and tumor tissue have been reported to be indicative of poor prognosis of breast cancer patients [108].

Intracellular mediators of VEGF induction

The intracellular signalling pathways that govern VEGF expression are still insufficiently explored, but apparently belong to the universal equipment of cells since VEGF expression is inducible in many different cell types. At present, a comprehensive assessment of several studies highlights the AP-1 transcription factor as an important common denominator for the regulation of VEGF expression. The VEGF promoter features four consensus motifs for AP-1 binding, and deletion analysis of the promoter suggests that the most proximal site is both sufficient and necessary for induction by TPA in transient transfection assays (A. Technau and D.M., unpublished data). As a rule, TPA stimulation of VEGF expression depends on protein synthesis, which is plausible because the Fos protein is not expressed in quiescent cells. Estrogen [86] as well as TGF β [109] also stimulate expression of Fos and Jun, the components of AP-1, by unknown mechanisms. On the other hand, glucocorticoids such as dexamethasone repress VEGF expression ([22,57], G. Finkenzeller, A. Technau, F. Totzke, and

D.M., manuscript submitted). The activated glucocorticoid receptor antagonizes AP-1 directed transcription by direct interaction with the Jun component of the AP-1 complex (reviewed in [110,111]). It remains to be tested, whether this type of transcriptional interference contributes to the anti-angiogenic property of steroid hormones (reviewed in [112]) or to the efficacy of dexamethasone in the treatment of rheumatoid arthritis and cerebral edema in connection with brain tumors. High expression of VEGF/VPF has been suggested to play an important role in the pathogenesis of both rheumatoid arthritis [113,114] and cerebral edema [42,47].

AP-1 is a classical nuclear target for PKC [115]. Direct pharmacological activation of PKC by phorbol esters leads to VEGF induction in many different cell types. PKC is a lipid regulated serine/threonine specific kinase, which is predominantly found in the cytoplasm but translocates to the membrane upon activation. Although originally defined as a biochemical entity, PKC has been revealed by molecular cloning as a multigene family consisting of eleven known isozymes. All tissues and cell types examined express one or more PKC isoforms, which seem to serve distinct biological functions (for a recent review see [116]). For example, overexpression of PKC δ in NIH 3T3 fibroblasts reduces the growth rate, while overexpression of PKC ϵ or η leads to malignant transformation [117]. PKC ϵ and η overexpressing NIH cells appear morphologically normal in culture, but grow in soft agar and readily form tumors in nude mice. Interestingly, these cells feature an enhanced and prolonged expression of VEGF in response to TPA *in vitro* (H. Mischak, F. Mushinski, J. Pierce, J. Goodnight, A.K., and W.K., unpublished data), prompting speculation that this trait contributes to tumorigenicity. Transformation and tumorigenicity elicited by PKC ϵ can be reversed by expression of a dominant negative Raf-1 mutant (A. Cacace, H. Mischak, M. Ueffing, B. Weinstein, and W.K., unpublished data), indicating that the Raf-1 kinase is a downstream signalling element of PKC ϵ . The Raf-1 kinase has been previously

implicated in the signal transduction of PKCs by the finding that PKC α can activate Raf-1 by direct phosphorylation [118-120]. In the cell, the Raf-1 kinase can also be activated by complex formation with Ras proteins [121-123, and references therein]. Both Ras and Raf-1 can stimulate AP-1 dependent transcription [124], and their oncogenic versions strongly induce VEGF expression in NIH 3T3 cells (S. Grugel, G. Finkenzeller, K. Weindel, B. Barleon, and D.M., manuscript submitted). In addition, Ras and certain tyrosine kinase oncogene proteins such as Src enhance the generation of diacylglycerol [125], which is the physiological activator of PKC. Signalling by Src-type tyrosine kinases, Ras, and PKC requires Raf-1 function [126,127]. Thus, Raf-1 might serve as a pivotal effector of an interdigitating network of membrane-associated signal transducers (Ras, Src, PKC), which collect and process a wide array of stimuli. Activated Raf initiates a kinase cascade by phosphorylating the Mek kinase, which in turn activates Erk/MAP kinases (reviewed in [77,128]). Erk can translocate to the nucleus, where it phosphorylates and activates transcription factors, amongst them c-Jun [129] and the ternary complex factor (TCF) [130], which induce immediate transcription of the *c-fos* gene. Thus, this pathway can regulate AP-1 by enhancing the transcriptional activity of the c-Jun protein and by affecting AP-1 composition due to induction of c-Fos expression. Activation of this pathway is required for oncogenic transformation of fibroblasts [131], and it is conceivable that the conversion to a tumorigenic phenotype might comprise activation of VEGF.

Another lead in respect to the regulation of VEGF expression was provided by the observation that the progression of dysplastic colon adenomas to carcinomas (reviewed in [132]) and of low malignancy astrocytomas to highly malignant glioblastomas [133] is accompanied by mutations in the p53 tumor suppressor gene as well as increased vascularization [35,41,42,45]. This correlation was corroborated by *in vitro* experiments with NIH cells expressing a temperature sensitive (ts) p53 protein [134]. At the

permissive temperature, where most of the p53 is in the mutant conformation, TPA-induced VEGF induction is dramatically and specifically augmented. This permissive effect of mutant p53 seems to be caused by the loss of a restraining influence of the wild-type p53 on VEGF induction, since VEGF expression is elevated in different cell lines where wildtype p53 has been genetically deleted or its function ablated by coexpression of the SV40 large T antigen or Mdm-2 (D.P. Dittmer, personal communication). Similarly, transformation of rat proximal tubule epithelial cells with a ts SV-40 large T antigen leads to VEGF induction at the permissive temperature. These findings are intriguing, because they link the regulation of VEGF expression and hence tumor vascularization to p53, the gene most commonly found mutated in human malignancies. 30%-50% of breast cancers carry p53 mutations [135]. Germline mutation of one p53 allele causes Li-Fraumeni syndrome [136,137], which predisposes to sarcomas as well as to early onset breast cancer eventually afflicting as many as 50% of female carriers.

Structure and function of the VEGF receptors

So far, two human receptor tyrosine kinases, Flt-1 (fms-like tyrosine kinase, [138]) and KDR (kinase-domain insert containing receptor, [139]), have been cloned and shown to function as specific receptors for VEGF. The homologous genes of KDR in mouse [140] and rat [141] have also been cloned and are called *flk-1* and *TKrC*, respectively. Putative avian homologues, Quek 1 and Quek 2 (for quail endothelial kinase), have been identified as well [142]. The two VEGF-R genes are closely related and together with the PDGF-R and the protooncogenes *c-fms* and *c-kit* belong to a class of tyrosine kinase receptors which is hallmarked by a kinase domain split by a large insert sequence. In contrast to PDGF-R, which contains five immunoglobulin-like domains, flt-1 and KDR possess seven immunoglobulin-like loops within their extracellular

portion. This property is shared with another receptor tyrosine kinase, Flt-4 [29,143], which does not bind VEGF and for which a ligand has not yet been identified [32]. A soluble splice variant of Flt-1 features only six immunoglobulin-like domains, but still can bind VEGF [144]. Both Flt-1 and KDR bind VEGF with high affinity [28,30]. When recombinantly produced in Sf9 cells they bind at least 3 VEGF isoforms (VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉) at comparable affinities (G.M.-B. and D.M., unpublished). Flt-1, but not Flk-1/KDR, can in addition bind placenta growth factor (PIGF), which is closely related to VEGF [145,146].

In regard to receptor function information is scarce. Porcine aortic ECs stably expressing human Flt-1 or KDR have been analyzed with respect to various cellular functions [147]. KDR expression leads to striking changes in cell morphology, actin reorganization, membrane ruffling, chemotaxis, and mitogenicity upon VEGF stimulation, whereas Flt-1 expressing cells lack such responses. Both Flt-1 and KDR autophosphorylate *in vitro* in response to VEGF, KDR much better than Flt-1. In intact cells only ligand-induced autophosphorylation of KDR is detectable. VEGF stimulation of Flt-1 or KDR expressing cells does not alter the activity of receptor-associated phosphatidylinositol-3-kinase or the tyrosine phosphorylation of phospholipase C- γ , but slightly increases the phosphorylation of GTPase activating (GAP) protein. In contrast, members of the Src-family, such as Fyn and Yes, show increased phosphorylation indicating activation.

Expression of VEGF receptors

VEGF-R expression in tissues and cell lines

As judged by Northern blot analysis, both receptors are expressed in a variety of tissues. *flt-1* mRNA is predominantly found in placenta, brain, heart, and lung [138]. The KDR mRNA is detected mainly in kidney, heart, spleen, and lung

[140]. At the cellular level, high affinity binding of VEGF has been almost exclusively demonstrated for ECs [27]. Comparison of Flt-1 and KDR expression in ECs has yielded overlapping yet distinct expression patterns [148]. The two known exceptions where VEGF receptor expression has been found in non-endothelial cells are monocytes (B. Barleon, A. Mantovani, and D.M., unpublished) and cells of the pancreatic tract [149].

VEGF-R expression during development

Evidence that the VEGF/VEGF receptor system plays a crucial part in vasculogenesis and angiogenesis comes from *in situ* hybridization studies with various fetal tissues from mice and humans [150,151]. High expression of both receptors is tightly controlled during embryogenesis and restricted to certain cell populations. Differential but overlapping expression of VEGF-R mRNAs is seen in all ECs, including the blood islands of the yolk sack from which the early progenitors of this lineage originate. *flk-1* transcripts can be found in the branching vessels of the developing and post-natal brain, but not in the adult brain [31]. The involvement of both receptors in vasculogenesis is strengthened by the finding that null mutations of either gene result in growth arrest of mice embryos between day 7.5 and 8.5 of embryonic development, soon after the cardiovascular system starts to form [152,153].

VEGF-R expression in pathological situations

In differentiated ECs in the adult, the amount of VEGF-R mRNA is remarkably reduced, but in various pathological situations activation and proliferation of ECs is accompanied by a drastic augmentation of VEGF-R expression. The most pronounced increase of VEGF-R expression has been reported for glioblastoma and gliosarcoma cells grafted on nude mice or syngeneic rats [37,38]. The ECs of the normal brain do not

express significant amounts of VEGF-R mRNA, whereas 24 out of 26 brain tumors examined expressed the *flt-1* and KDR genes at various levels [42]. No obvious correlation was found with VEGF mRNA levels investigated in the same tissues. Another study revealed that adenocarcinomas express both types of VEGF-R in vascular ECs, while adjacent healthy tissue shows no response [45]. Similarly, no *flt-1* transcripts can be found in the normal skin endothelium, but *flt-1* expression is greatly enhanced during wound healing with maximal expression at day 7 after wounding [53]. Taken together, these findings indicate that in highly vascularized tumors there is considerable expression of both VEGF and VEGF-Rs. As in the case of VEGF, the interesting question concerning the regulation of VEGF receptor expression is: What are the mechanisms which enhance *flt-1* and KDR expression? The answer to this question as well as to the question concerning the mechanisms of VEGF gene transcription is a major issue pertaining to the understanding of the development and progression of solid tumors.

Regulation of VEGF-R gene expression

The turnover rates of vascular ECs in the healthy adult human organism are generally very low. A marked exception is the female reproductive system, where the need for additional vasculature is constantly imposed by the cyclic evolution of transient tissue structures. Therefore, it is not astonishing that the cyclic expression of both VEGF and VEGF-Rs is observed in tissues involved in this process [81]. As mentioned above, in certain pathological situations such as the development of solid tumors, the expression of VEGF in tumor cells and VEGF receptors in ECs adjacent to and growing into the tumor is characteristic for the disease. Very little is known about the regulation of the expression of the VEGF-R genes. Only recently, a few growth factors and cytokines have been shown to modulate *flt-1* and KDR gene expression [154]. bFGF causes a sig-

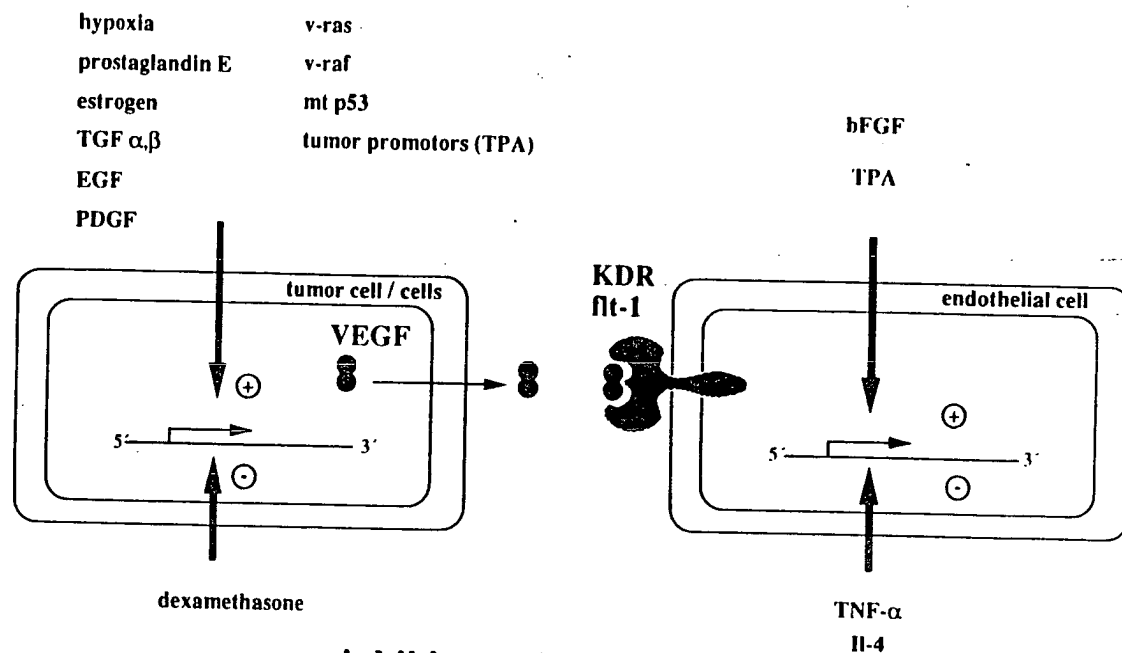
nificant increase in *flt-1* mRNA and a slight increase in KDR mRNA in human umbilical vein ECs. Interestingly, bFGF is produced by some tumors ([155] and references therein). The cytokine IL-4 slightly upregulates *flt-1* expression, while repressing KDR expression. TNF α strongly inhibits the expression of both receptors. Furthermore, the tumor promoter TPA induces robust *flt-1* expression, whereas KDR expression is almost unaffected. VEGF itself (at least the two isoforms VEGF₁₆₅ and VEGF₁₂₁) is not able to augment the expression of either receptor. The same seems to be true for PlGF-1 and PlGF-2, the two isoforms of PlGF. The challenge clearly is to identify the factor(s) responsible for the high expression of VEGF-Rs observed in ECs lining tumor vessels. Another important issue is the cell specific expression of the VEGF-R receptors. Cloning of the promoters for the two receptor genes will certainly further our insight into both processes: cell-specific expression and factor-dependent enhancement of expression.

Outlook

Given the mounting evidence that VEGF is one of the most important factors governing tumor angiogenesis, it is imperative to learn more about the molecular mechanisms which control the expression of VEGF in the tumor cells, and which regulate the expression of the VEGF-R in the ECs. The available data are summarized in Figure 1.

Two pioneering experiments, one showing suppression of experimental tumors by neutralizing anti-VEGF antibodies [49,50], the other by expression of a dominant negative *flt-1* mutant [51], have in principle installed the inhibition of VEGF or its receptors as a valid concept for a novel approach to tumor therapy. A major advantage seems the apparent lack of severe toxic side effects observed in these experiments. Furthermore, because of the potential of VEGF to permeabilize the vascular wall, there is a chance to interfere with metastasis formation. This, however, remains to be evaluated in appropriate *in*

enhancers of gene expression



inhibitors of gene expression

Figure 1. Regulation of VEGF and VEGF-R gene expression

vivo systems. A promising task for the future will be to develop low molecular weight substances to inhibit the expression or function of VEGF and its receptors which are better suited for use in the clinic than antibodies or dominant negative genes.

Despite the recent advances many questions remain to be answered. Are both VEGF-Rs involved in tumor angiogenesis? Do additional VEGF-Rs exist? How is their expression regulated? Are all known VEGF isoforms tumor angiogenic factors? Which side effects are to be expected when the VEGF/VEGF receptor system is blocked? Although the expression of VEGF-Rs has been reported for almost all solid tumors investigated so far and the high expression of VEGF-Rs in tumor blood vessels has been successfully shown at least in a few tumor types, the question remains: Is VEGF the only or at least the most important tumor angiogenesis factor, or are there comparable ligand/receptor systems which

govern tumor angiogenesis? Recently, a new family of receptor tyrosine kinases has been found to be predominantly expressed in ECs [156]. The ligands for these receptors have not been identified yet. Preliminary studies show that two members of this receptor family, tie-1 and tie-2, are expressed in human glioblastomas (K. Weindel and D.M., unpublished), but more work has to be done to elucidate the role of these receptors in tumor angiogenesis.

References

1. Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82:4-7, 1990
2. Folkman J: The role of angiogenesis in tumor growth. *Semin Cancer Biol* 3:65-71, 1992
3. Fox SB, Leek RD, Smith K, Hollyer J, Greenall M, Harris AL: Tumor angiogenesis in node-negative

- breast carcinomas: relationship with epidermal growth factor receptor, estrogen receptor, and survival. *Breast Cancer Res Treat* 29:109-116, 1994
4. Weidner N, Semple P, Welch W, Folkman J: Tumor angiogenesis and metastasis. Correlation in invasive breast carcinoma. *New Engl J Med* 324:1-6, 1991
 5. Horak ER, Leek R, Klenk N, LeJeune S, Smith K, Stuart N, Greenall M, Stepniowska K, Harris AL: Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 340:1120-1124, 1992
 6. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G: Tumor angiogenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 84:1875-1888, 1992
 7. Fidler IJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185-188, 1994
 8. Bicknell R, Harris AL: Anticancer strategies involving the vasculature: vascular targeting and the inhibition of angiogenesis. *Seminars in Cancer Biology* 3:399-407, 1992
 9. Klagsbrun M, Soker S: VEGF/VPS: the angiogenesis factor found? *Current Biology* 3:699-702, 1993
 10. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266:11947-11954, 1991
 11. Charnock Jones DS, Sharkey AM, Rajput Williams J, Burch D, Schofield JP, Fountain SA, Boock CA, Smith SK: Identification and localization of alternatively spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. *Biol Reprod* 48:1120-1128, 1993
 12. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 5:1806-1814, 1991
 13. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851-858, 1989
 14. Gospodarowicz D, Abraham JA, Schilling J: Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc Natl Acad Sci USA* 86:7311-7315, 1989
 15. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Epley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 84:1470-1478, 1989
 16. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246:1309-1312, 1989
 17. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306-1309, 1989
 18. Senger DR, Van de Water L, Brown LF, Nagy JA, Yeo KT, Yeo TK, Berse B, Jackman RW, Dvorak AM, Dvorak HF: Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev* 12:303-324, 1993
 19. Nagy JA, Brown LF, Senger DR, Lanir N, Van De Water L, Dvorak AM, Dvorak HF: Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochim Biophys Acta* 948:305-326, 1988
 20. Dvorak HF, Nagy JA, Berse B, Brown LF, Yeo K, Yeo T, Dvorak AM, Van De Water L, Sioussat TM, Senger DR: Vascular permeability factor, fibrin, and the pathogenesis of tumor stroma function. *Ann NY Acad Sci* 667:101-111, 1992
 21. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan YC, Olander JV, Connolly DT, Stern D: Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity and promotes monocyte migration. *J Exp Med* 172:1535-1545, 1990
 22. Iijima K, Yoshikawa N, Connolly DT, Nakamura H: Human mesangial cells and peripheral blood mononuclear cells produce vascular permeability factor. *Kidney Int* 44:959-966, 1993
 23. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C: Macrophages and angiogenesis. *J Leukoc Biol* 55:410-422, 1994
 24. Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly D, Stern D, Kao J: Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. *Blood* 81:2767-2773, 1993
 25. Vaisman N, Gospodarowicz D, Neufeld G: Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem* 265:19461-19466, 1990
 26. Plouet J, Moukadi H: Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. *J Biol Chem* 265:22071-22074, 1990
 27. Olander JV, Connolly DT, DeLarco JE: Specific binding of vascular permeability factor to endothelial cells. *Biochem Biophys Res Commun* 175:68-76,

- 1991
28. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989-991, 1992
29. Galland F, Karamysheva A, Pebusque M, Borg J, Rottapel R, Dubreuil P, Rosnet O, Birnbaum D: The flt4 gene encodes a transmembrane tyrosine kinase related to the vascular endothelial growth factor receptor. *Oncogene* 8:1233-1240, 1993
30. Terman BI, Dougher Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 187:1579-1586, 1992
31. Millauer B, Witzigmann Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835-846, 1993
32. Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L, Alitalo K: Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene* 9:3545-3555, 1994
33. Senger DR, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 46:5629-5632, 1986
34. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van De Water L, Senger DR: Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J Exp Med* 174:1275-1278, 1991
35. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, Robertson JT, Ali IU, Oldfield EH: Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. *J Clin Invest* 91:153-159, 1993
36. Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 3:211-220, 1992
37. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 359:845-848, 1992
38. Plate KH, Breier G, Millauer B, Ullrich A, Risau W: Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res* 53:5822-5827, 1993
39. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van de Water L, Senger DR: Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J Exp Med* 174:1275-1278, 1991
40. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C: Macrophages and angiogenesis. *J Leukoc Biol* 55:410-422, 1994
41. Alvarez JA, Baird A, Tatum A, Daucher J, Chorsky R, Gonzalez AM, Stopa EG: Localization of basic fibroblast growth factor and vascular endothelial growth factor in human glial neoplasms. *Mod Pathol* 5:303-307, 1992
42. Weindel K, Moringlane JR, Marme D, Weich HA: Detection and quantification of vascular endothelial growth factor/vascular permeability factor in brain tumor tissue and cyst fluid: the key to angiogenesis? *Neurosurgery* 35:439-449, 1994
43. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, Senger DR: Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am J Pathol* 143:1255-1262, 1993
44. Takahashi A, Sasaki H, Kim SJ, Tobisu K, Kakizoe T, Tsukamoto T, Kumamoto Y, Sugimura T, Terada M: Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 54:4233-4237, 1994
45. Brown LF, Berse B, Jackman R, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 53:4727-4735, 1993
46. Toi M, Hoshima S, Takayanagi T, Tominaga T: Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 85:1045-1049, 1994
47. Criscuolo GR: The genesis of peritumoral vasogenic brain edema and tumor cysts: a hypothetical role for tumor-derived vascular permeability factor. *Yale J Biol Med* 66:277-314, 1993
48. Ferrara N, Winer J, Burton T, Rowland A, Siegel M, Phillips HS, Terrell T, Keller GA, Levinson AD: Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells. *J Clin Invest* 91:160-170, 1993

- 2-5827, 1993
erse B, Nagy
ter L, Senger
ability factor
in tumors:
J Exp Med
- M, Bhardwaj
sis. J Leukoc
- er J, Chorsky
tion of basic
endothelial
Mod Pathol
- Weich HA:
endothelial
tor in brain
ngiogenesis?
- azzi K, Man-
Increased
tor (vascular
ors in kidney
1255-1262,
- K, Kakizoe
ra T, Terada
messenger
factor and
carcinoma
es 54:4233-
- ognazzi K,
expression of
endothelial
ocarcinomas
es 53:4727-
- ominaga T:
rowth factor
d with early
Cancer Res
- al vasogenic
ical role for
tor. Yale J
- A, Siegel M,
vinson AD:
a factor does
s a growth
ary cells. J
49. Kondo S, Asano M, Suzuki H: Significance of vascular endothelial growth factor/vascular permeability factor for solid tumor growth, and its inhibition by the antibody. *Biochem Biophys Res Commun* 194: 1234-1241, 1993
50. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362:841-844, 1993
51. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A: Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature* 367:576-579, 1994
52. Breier G, Albrecht U, Sterrer S, Risau W: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114:521-532, 1992
53. Peters KG, De Vries C, Williams LT: Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci USA* 90:8915-8919, 1993
54. Risau W: Angiogenesis and endothelial cell function. *Arzneimittelforschung* 44:416-417, 1994
55. Jakeman LB, Armanini M, Phillips HS, Ferrara N: Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology* 133:848-859, 1993
56. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest* 89:244-253, 1992
57. Harada S, Nagy JA, Sullivan KA, Thomas KA, Endo N, Rodan GA, Rodan SB: Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. *J Clin Invest* 93:2490-2496, 1994
58. Midy V, Plouet J: Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. *Biochem Biophys Res Commun* 199:380-386, 1994
59. Castellot JJ Jr, Kambe AM, Dobson DE, Spiegelman BM: Heparin potentiation of 3T3-adipocyte stimulated angiogenesis: mechanisms of action on endothelial cells. *J Cell Physiol* 127:323-329, 1986
60. Dobson DE, Kambe A, Block E, Dion T, Lu H, Castellot JJ Jr, Spiegelman BM: 1-butylglycerol: a novel angiogenesis factor secreted by differentiating adipocytes. *Cell* 61:223-230, 1990
61. Green H, Kehinde O: Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J Cell Physiol* 101:169-171, 1979
62. Claffey KP, Wilkison WO, Spiegelman BM: Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem* 267:16317-16322, 1992
63. Kinasewitz GT, Groome LJ, Marshall RP, Leslie WK, Diana JN: Effect of hypoxia on permeability of pulmonary endothelium of canine visceral pleura. *J Appl Physiol* 61:554-560, 1986
64. Olesen SP: Rapid increase in blood-brain barrier permeability during severe hypoxia and metabolic inhibition. *Brain Res* 368:24-29, 1986
65. Adair TH, Gay WJ, Montani JP: Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am J Physiol* 259:R393-R404, 1990
66. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-845, 1992
67. Goldberg MA, Schneider TJ: Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 269:4355-4359, 1994
68. Brogi E, Wu T, Namiki A, Isner JM: Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation* 90:649-652, 1994
69. Ladoux A, Frelin C: Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem Biophys Res Commun* 195: 1005-1010, 1993
70. Rondon JJ, MacMillan LA, Beckman BS, Goldberg MA, Schneider T, Bunn HF, Malter JS: Hypoxia upregulates the activity of a novel erythropoietin mRNA binding protein. *J Biol Chem* 266:16594-16598, 1991
71. Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ: Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proc Natl Acad Sci USA* 91:6496-6500, 1994
72. McClure N, Healy DL, Rogers PA, Sullivan J, Beaton L, Haning RV Jr, Connolly DT, Robertson DM: Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. *Lancet* 344:235-236, 1994
73. Webster KA, Discher DJ, Bishopric NH: Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxic cardiac myocytes. *J Biol Chem* 268: 16852-16858, 1993
74. Curran T, Franza BR: Fos and Jun: The AP-1 connection. *Cell* 55:395-397, 1988
75. Koong AC, Chen EY, Kim CY, Giaccia AJ: Activators of protein kinase C selectively mediate cellular cytotoxicity to hypoxic cells and not aerobic cells.

- Int J Radiat Oncol Biol Phys 29:259-265, 1994
76. Eckardt KU, Ring A, Maier M, Gess B, Fabbro D, Kurtz A: Hypoxia-induced accumulation of erythropoietin mRNA in isolated hepatocytes is inhibited by protein kinase C. *Pflugers Arch* 426:21-30, 1994
77. Davis RJ: MAPKs: new JNK expands the group. *Trends in Biochem Sci* 19:470-473, 1994
78. Findlay JK: Angiogenesis in reproductive tissues. *J Endocrinol* 111:357-366, 1986
79. Christianes GCM, Sixma JJ, Haspels AA: Hemostasis in menstrual endometrium: a review. *Obstet Gynecol Survey* 37:281-303, 1982
80. Cullinan Bove K, Koos RD: Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* 133: 829-837, 1993
81. Shweiki D, Itin A, Neufeld G, Gitay Goren H, Keshet E: Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 91:2235-2243, 1993
82. Phillips HS, Hains J, Leung DW, Ferrara N: Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 127:965-967, 1990
83. Garrido C, Saule S, Gospodarowicz D: Transcriptional regulation of vascular endothelial growth factor gene expression in ovarian bovine granulosa cells. *Growth Factors* 8:109-117, 1993
84. Imagawa M, Chiu R, Karin M: Transcription factor AP2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* 51:251-260, 1987
85. Aronica SM, Kraus WL, Katzenellenbogen BS: Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* 91:8517-8521, 1994
86. Weisz A, Cicatiello L, Persicot E, Scalone M, Bresciani F: Estrogen stimulates transcription of c-jun protooncogene. *Mol Endocrinol* 4:1041-1050, 1990
87. Krasil'nikov MA, Shatskaya VA, Kuzmina ZV, Barinov VV, Letyagin VP, Bassalyk LS: Regulation of phospholipid turnover by steroid hormones in endometrial carcinoma and breast cancer cells. *Acta Endocrinol Copenh* 128:543-548, 1993
88. Etindi RN, Manni A, Martel J: The effects of TGF- α and 17 β -estradiol on polyphosphoinositide metabolism in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 24:61-70, 1992
89. Cantley L: Lipid second messengers. *Cell* 77:329-334, 1994
90. Migliaccio A, Pagano M, Auricchio F: Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7 cells. *Oncogene* 8:2183-2191, 1993
91. Detmar M, Brown LF, Claffey KP, Yeo KT, Kocher O, Jackman RW, Berse B, Dvorak HF: Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* 180:1141-1146, 1994
92. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor- β in fibroblastic and epithelial cells. *J Biol Chem* 269:6271-6274, 1994
93. Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY: Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* 4:121-133, 1993
94. Finkenzeller G, Marme D, Weich HA, Hug H: Platelet-derived growth factor-induced transcription of the vascular endothelial growth factor gene is mediated by protein kinase C. *Cancer Res* 52:4821-4823, 1992
95. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al: Transforming growth factor type β : rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci USA* 83:4167-4171, 1986
96. Heimark RL, Twardzik DR, Schwartz SM: Inhibition of endothelial regeneration by type- β transforming growth factor from platelets. *Science* 233:1078-1080, 1986
97. Baird A, Durkin T: Inhibition of endothelial cell proliferation by type β -transforming growth factor: interactions with acidic and basic fibroblast growth factors. *Biochem Biophys Res Commun* 138:476-482, 1986
98. Walker RA, Dearing SJ, Gallacher B: Relationship of transforming growth factor β 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. *Br J Cancer* 69:1160-1165, 1994
99. Murray PA, Barrett Lee P, Travers M, Luqmani Y, Powles T, Coombes RC: The prognostic significance of transforming growth factors in human breast cancer. *Br J Cancer* 67:1408-1412, 1993
100. Goto F, Goto K, Weindel K, Folkman J: Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels [see comments]. *Lab Invest* 69:508-517, 1993

101. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT: Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 92:2569-2576, 1993
102. Stewart AJ, Westley BR, May FE: Modulation of the proliferative response of breast cancer cells to growth factors by oestrogen. *Br J Cancer* 66:640-648, 1992
103. Leon SP, Zhu J, Black PM: Genetic aberrations in human brain tumors. *Neurosurgery* 34:708-722, 1994
104. Chrysogelos SA, Dickson RB: EGF receptor expression, regulation, and function in breast cancer. *Breast Cancer Res Treat* 29:29-40, 1994
105. Normanno N, Ciardiello F, Brandt R, Salomon DS: Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. *Breast Cancer Res Treat* 29:11-27, 1994
106. Klijn JG, Look MP, Portengen H, Alexieva Figusch J, van Putten WL, Foekens JA: The prognostic value of epidermal growth factor receptor (EGF-R) in primary breast cancer: results of a 10 year follow-up study. *Breast Cancer Res Treat* 29:73-83, 1994
107. McCormick F: Signal transduction. How receptors turn Ras on. *Nature* 363:15-16, 1993
108. Seymour L, Dajee D, Bezwoda WR: Tissue platelet derived-growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 26:247-252, 1993
109. Pertovaara L, Sistonen L, Bos TJ, Vogt PK, Keski Oja J, Alitalo K: Enhanced jun gene expression is an early genomic response to transforming growth factor beta stimulation. *Mol Cell Biol* 9:1255-1262, 1989
110. Konig H, Ponta H, Rahmsdorf HJ, Herrlich P: Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. *EMBO J* 11:2241-2246, 1992
111. Ponta H, Cato AC, Herrlich P: Interference of pathway specific transcription factors. *Biochim Biophys Acta* 1129:255-261, 1992
112. Folkman J, Ingber D: Inhibition of angiogenesis. *Semin Cancer Biol* 3:89-96, 1992
113. Fava RA, Olsen NJ, Spencer Green G, Yeo KT, Yeo TK, Berse B, Jackman RW, Senger DR, Dvorak HF, Brown LF: Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J Exp Med* 180:341-346, 1994
114. Koch AE, Harlow LA, Haines GK, Amento EP, Unemori EN, Wong WL, Pope RM, Ferrara N: Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 152:4149-4156, 1994
115. Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P: 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. *Mol Cell Biol* 7:2256-2266, 1987
116. Goodnight J, Mushinski JF, Mischak H: Protein kinase C in differentiation and transformation. *Adv Cancer Res* 64:160-209, 1994
117. Mischak H, Goodnight J, Kolch W, Martiny-Baron G, Schaechtele C, Kazanietz MG, Blumberg PM, Pierce JH, Mushinski JF: Overexpression of PKC δ and - ϵ in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence and tumorigenicity. *J Biol Chem* 268:6090-6096, 1993
118. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR: PKC α activates Raf-1 by direct phosphorylation. *Nature* 364:249-251, 1993
119. Sözeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark GE III, Stabel S: Activation of the c-Raf protein kinase by protein kinase C phosphorylation. *Oncogene* 7:2259-2262, 1992
120. Carroll MP, May WS: Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. *J Biol Chem* 269:1249-1256, 1994
121. Hallberg B, Ryter SI, Downward J: Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *J Biol Chem* 269:3913-3916, 1994
122. Koide H, Satoh K, Nakafuku M, Kaziro Y: GTP-dependent association of Raf-1 with Ha-Ras: identification of Ras as a target downstream of Ras in mammalian cells. *Proc Natl Acad Sci USA* 90:8683-8686, 1993
123. Moodie SA, Willumsen BM, Weber MJ, Wolfman A: Complexes of Ras/GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 260:1658-1661, 1993
124. Bruder JT, Heidecker G, Rapp UR: Serum-, TPA-, and Ras-induced expression from AP-1/Ets-driven promoters requires Raf-1 kinase. *Genes Dev* 6:545-556, 1992
125. Diaz-Laviada I, Larrodera P, Diaz-Meco MC, Cornet ME, Guttal PM, Johansen T, Moscat J: Evidence for a role of phosphatidylcholine-hydrolyzing phospholipase C in the regulation of protein kinase C by ras and src oncogenes. *EMBO J* 9:3907-3912, 1990
126. Kolch W, Heidecker G, Lloyd P, Rapp UR: Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. *Nature* 349:426-428, 1991
127. Kolch W, Heidecker G, Troppmair J, Yanagihara K,

- Bassin RH, Rapp UR: Raf revertant cells resist transformation by non-nuclear oncogenes and are deficient in the induction of early response genes by TPA and serum. *Oncogene* 8:361-370, 1993
128. Egan SE, Weinberg RA: The pathway to signal achievement. *Nature* 365:781-783, 1993
 129. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR: Phosphorylation of c-jun mediated by MAP kinases. *Nature* 353:670-674, 1991
 130. Gille HG, Sharrocks AD, Shaw PE: Phosphorylation of transcription factor p62^{TCF} by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* 358:414-417, 1992
 131. Troppmair J, Bruder JT, Munoz H, Lloyd PA, Kyriakis J, Banerjee P, Avruch J, Rapp UR: Mitogen-activated protein kinase / extracellular signal-regulated kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. *J Biol Chem* 269:7030-7035, 1994
 132. Vogelstein B, Kinzler KW: The multistep nature of cancer. *Trends Genet* 9:138-141, 1993
 133. Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein B: Clonal expansion of p53 mutant cells is associated with brain tumour progression. *Nature* 355:846-847, 1992
 134. Kieser A, Weich HA, Brandner G, Marme D, Kolch W: Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 9:963-969, 1994
 135. Smith HS, Lu Y, Deng G, Martinez O, Krams S, Ljung BM, Thor A, Lagios M: Molecular aspects of early stages of breast cancer progression. *J Cell Biochem Suppl* 17G:144-152, 1993
 136. Li FP, Fraumeni JF Jr: Soft tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome. *Ann Intern Med* 71:747-752, 1969
 137. Malkin D, Li FP, Strong LC, Fraumeni JF, Nelson CE, Kim DH, Kassell J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 250:1233-1238, 1990
 138. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushima H, Sato M: Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene* 5:519-524, 1990
 139. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB: Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* 6:1677-1683, 1991
 140. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR: A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci USA* 88:9026-9030, 1991
 141. Sarzani R, Arnaldi G, De Pirro R, Moretti P, Schiaffino S, Rappelli A: A novel endothelial tyrosine kinase cDNA homologous to platelet-derived growth factor receptor cDNA. *Biochem Biophys Res Commun* 186:706-714, 1992
 142. Eichmann A, Marcelle C, Breant C, Le Douarin NM: Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech Dev* 42:33-48, 1993
 143. Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Pertovaara L, Alitalo R, Alitalo K: FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines [published erratum appears in *Cancer Res* 53:3845, 1993]. *Cancer Res* 52:5738-5743, 1992
 144. Kendall RL, Thomas KA: Inhibition of vascular endothelial growth factor by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* 90:10705-10709, 1993
 145. Park JE, Chen HH, Winer J, Houck KA, Ferrara N: Placenta growth factor. *J Biol Chem* 269:25646-25654, 1994
 146. Kendall RL, Wang G, DiSalvo J, Thomas KA: Specificity of vascular endothelial cell growth factor receptor ligand binding domains. *Biochem Biophys Res Commun* 201:326-330, 1994
 147. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH: Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269:26988-26995, 1994
 148. Barleon B, Hauser S, Schollmann C, Weindel K, Marme D, Yayon A, Weich HA: Differential expression of the two VEGF receptors flt and KDR in placenta and vascular endothelial cells. *J Cell Biochem* 54:56-66, 1994
 149. Oberg C, Waltenberger J, Claesson Welsh L, Welsh M: Expression of protein tyrosine kinases in islet cells: possible role of the Flk-1 receptor for beta-cell maturation from duct cells. *Growth Factors* 10:115-126, 1994
 150. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT: Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 90:7533-7537, 1993
 151. Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J: flk-1, an flt-related receptor tyrosine kinase, is an early marker for endothelial cell precursors. *Development* 118:489-498, 1993
 152. Fong G, Rossant J, Breitman ML: Requirement of

- the VEGF receptor Flt-1 for the normal embryonic development. VIIIth Intern Symp on the Biology of Vascular Cells, Abstracts, 1994
153. Shalaby F, Rossant J, Breitman ML, Schuh AC: The role of flk-1 in endothelial cell development. VIIIth Intern Symp on the Biology of Vascular Cells, Abstracts, 1994
 154. Barleon B, Weich HA, Martiny-Baron G, Marme D: Control of the two VEGF receptor genes KDR and flt-1 by cytokines and growth factors. *Cancer Res*, submitted
 155. Soutter AD, Nguyen M, Watanabe H, Folkman J: Basic fibroblast growth factor secreted by an animal tumor is detectable in urine. *Cancer Res* 53:5297-5299, 1993
 156. Sato TN, Qin Y, Kozak CA, Audus KL: Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA* 90:9355-9358, 1993

Pericyte involvement in capillary sprouting during angiogenesis in situ

Volker Nehls, Kristin Denzer, and Detlev Drenckhahn

Anatomisches Institut der Universität, Koellikerstr. 6, W-8700 Würzburg, Germany

Received June 9, 1992 / Accepted September 7, 1992

Summary. To investigate the participation of microvascular pericytes in the process of capillary sprouting, we examined whole-mount preparations of the rat mesentery by use of a double immunofluorescence approach. Angiogenesis was induced by intraperitoneal injections of either the mast cell-degranulating substance compound 48/80 or tumor cell-conditioned medium. Capillary sprouts were visualized by staining with rhodamin-conjugated phalloidin and pericytes were simultaneously stained by an antibody to the intermediate filament protein desmin. Developing pericytes were negative for the smooth-muscle isoform of α -actin, but were clearly reactive for desmin. Pericytes appear to be involved in the earliest stages of capillary sprouting. Pericytes were regularly found lying at and in front of the advancing tips of endothelial sprouts. At many sites pericytes were seen to bridge the gap between the leading edges of opposing endothelial sprouts, which were apparently preparing to merge, suggesting that pericytic processes may serve as guiding structures aiding outgrowth of endothelial cells.

Key words: Pericytes – Angiogenesis – Capillaries – Capillary sprouting – Desmin – Immunocytochemistry – Rat – Adenocarcinoma cells, rat

Pericytes have been suggested to suppress endothelial proliferation (Orlidge and D'Amore 1987) without having any obvious role during the initial phase of capillary sprouting (Paweletz and Knierim 1989; Blood and Zetter 1990; Folkman and Klagsbrun 1987; Crocker et al. 1970). Several morphological studies indicate that at least a fraction of capillary pericytes may evolve from perivascular fibroblasts (Sims 1986; Crocker et al. 1970; Rhodin and Fujita 1989; Nakayasu 1988). Final proof for this theory, however, is lacking. We have recently shown that pericytes located at the midcapillary segments of the microvascular tree do not contain the α -isoform of smooth-muscle type actin (Sm α -actin; Nehls and Drenckhahn 1991a). In contrast, pericytes of pre- and postcapillaries were distinctly positive for Sm α -actin. As typical fibroblasts also do not contain Sm α -actin (Sappino et al. 1990), this observation may be tentatively interpreted in terms of a fibroblastic nature of midcapillary pericytes. To investigate closer the development of pericytes during capillary sprouting we examined the angiogenic reaction occurring after intraperitoneal application of compound 48/80 (Norrby et al. 1986) and tumor-cell conditioned medium, respectively. We provide further evidence for a fibroblastic origin of pericytes and, more importantly, for an early involvement of pericytes in capillary sprouting.

Materials and methods

Induction of angiogenesis

Mast cell-stimulated angiogenesis was induced by daily intraperitoneal injections of compound 48/80 (Sigma, Deisenhofen, FRG) over a range of 5 days, as described earlier (Norrby et al. 1986). At different intervals after the first injection, during the incremental phase of capillary sprouting or after complete capillary loops have been formed, the animals were sacrificed and mesenteries were mounted on teflon rings. As an alternative angiogenesis system, conditioned medium of tumor cells was injected into the peritoneal cavity of rats. Walker 256 carcinoma cells (rat adenocarcinoma cells) were generously provided by the Deutsches Krebsforschungs-

Angiogenesis, the development of new blood vessels from a pre-existing microvascular network, is of fundamental importance for several physiological and pathological events such as organ development and tumor growth (Paweletz and Knierim 1989; Blood and Zetter 1990; Folkman and Klagsbrun 1987). Endothelial cells have been shown by in vitro studies to be capable of forming random networks of capillary-like endothelial tubes (Folkman and Haudenschild 1980; Pepper et al. 1990). Capillaries in situ contain a further cell type, the pericyte, which is found enclosed within the microvascular basal lamina (Sims 1986; Diaz-Flores et al. 1991).

zentrum, Heidelberg, FRG and grown in RPMI 1640 medium (Gibco, Eggenstein, FRG) supplemented with 10% fetal calf serum (FCS, Gibco) at a density of 7×10^5 cells/ml. Cells were centrifuged at $1000 \times g$ for 10 min and supernatants were stored frozen at -20°C . One ml of the tumor cell-conditioned medium was injected twice daily over a range of 10 days. At day 11 the rats were sacrificed and mesenteries processed for examination by immunofluorescence.

Immunofluorescence on whole-mount preparations

Rat mesenteries were mounted on teflon rings and fixed in 2% formaldehyde in phosphate-buffered saline (140 mM NaCl, 10 mM Na phosphate, pH 7.3, PBS) for 5 min. The mesothelial layer was removed by controlled proteolytic digestion in 0.5% Dispase II (Boehringer, Mannheim, FRG) as described previously (Nehls and Drenckhahn 1991a; Nehls and Drenckhahn 1991b). After permeabilization in acetone at -20°C samples were incubated with first antibodies for 2 h at room temperature and, following extensive rinsing, in fluorescein-isothiocyanate (FITC) and tetramethyl rhodamyl-isothiocyanate (TRITC)-conjugated second antibodies. FITC-labeled goat anti-rabbit IgG and TRITC-labeled goat anti-mouse IgG were from Bayer Diagnostic, Munich, FRG. For double fluorescence purposes, a mixture of both second antibodies was used. In case of double-labeling with antibodies and TRITC-phalloidin, tissue samples were first incubated with primary and secondary antibodies and then stained for filamentous actin (F-actin) by 30-min incubation with $5 \mu\text{g/ml}$ TRITC-phalloidin and appropriate controls as described (Nehls and Drenckhahn 1991b; Drenckhahn et al. 1984).

Antibodies

The following antibodies were used in this study: Monoclonal mouse IgG against the NH_2 -terminal decapeptide of smooth muscle α -actin (SM α -actin, Progen, Heidelberg, FRG) (Skalli et al. 1986). The antibody to chicken gizzard desmin was raised in a rabbit and was affinity-purified according to standard procedures (Drenckhahn and Franz 1986). In immunoblot analyses, the antibody showed strong binding to desmin (53 kD band) of smooth muscle cells. There was no detectable cross-reactivity to vimentin (negative results with endothelial cells). The antibody to factor VIII-related antigen (human von Willebrand factor) was from Sigma, Deisenhofen, FRG. Double immunostaining of capillaries with both the antibody to desmin and anti-factor VIII-related antigen clearly revealed that endothelial cells were left completely unstained by anti-desmin (not shown).

Results

Angiogenesis in the rat mesentery

The translucent parts of the membranous mesentery of normal rats are essentially free of blood vessels (Norrby et al. 1986). To study the cellular events in the process of capillary sprouting, angiogenesis has to be induced by angiogenic stimuli. For this purpose, we used the model of mast cell-stimulated angiogenesis which was introduced by Norrby et al. (1986). After repeated intraperitoneal injections of the mast cell-degranulating substance 48/80, a dense capillary network developed throughout the stromal layer of the mesentery. These microvessels could be visualized clearly by staining of

filamentous actin with rhodamin-tagged phalloidin (Fig. 1a). To investigate whether the cellular events detected in mast cell-induced capillary sprouting are comparable to other types of angiogenesis, we repeatedly injected Walker 256 carcinoma cell-conditioned medium into the peritoneal cavity of rats. The ensuing vascular growth reaction appeared to be less intense as compared with mast cell-induced angiogenesis. However, there were always sufficient numbers of capillary sprouts, allowing to investigate the fate of pericytes during capillary sprouting.

Immunostaining of microvascular cells

The most ideally suited antibody for selective staining of microvascular pericytes appeared to be an affinity-purified antibody raised against chicken gizzard desmin (Fig. 1b, c). This antibody displayed strong binding to pericytes of all microvascular segments as well as to vascular smooth muscle cells. Endothelial cells and quiescent fibroblasts were left completely unstained. In contrast, the monoclonal antibody to SM α -actin failed to stain pericytes of capillaries and capillary sprouts including young capillary loops (Fig. 2a, b). By these criteria (positive for desmin, negative for SM α -actin) young pericytes could be safely distinguished from any other cell type of the mesentery. The nascent microvasculature exhibited positive staining for SM α -actin only on proximal vascular segments which by their relatively large diameters and branching pattern were identified as pre- and postcapillaries according to the definition given previously (Nehls and Drenckhahn 1991a; Nehls and Drenckhahn 1991b) (Fig. 2a, b).

For positive identification of endothelial cells we used in a first attempt antibodies to the factor-VIII related antigen. This antibody was bound to numerous small cytoplasmic granules, most likely Weibel-Palade bodies. These were mainly located in the perinuclear region of endothelial cells (Fig. 2d). Unfortunately, the cell margins and the utmost distal endings of endothelial sprouts were left unstained (Fig. 2d). TRITC-phalloidin, in contrast, exhibited strong binding to the total endothelial cell body including the cell margins and distal extensions of endothelial sprouts (Fig. 2c). Pericytes in close apposition to the capillary wall were also stained by phalloidin, whereas immature pericytes (defined as desmin-positive cells lying adjacent to capillary sprouts), displayed only weak to moderate fluorescence (Fig. 3). Fibroblasts were rather weakly labeled by TRITC-phalloidin, most probably due to lower amounts of F-actin in these cells (Fig. 3).

Capillary sprouting

Double-fluorescent imaging of capillary sprouts by TRITC-phalloidin and anti-desmin revealed that pericytes were regularly present at the leading tips of endothelial sprouts (Fig. 3). This occurred irrespective of the

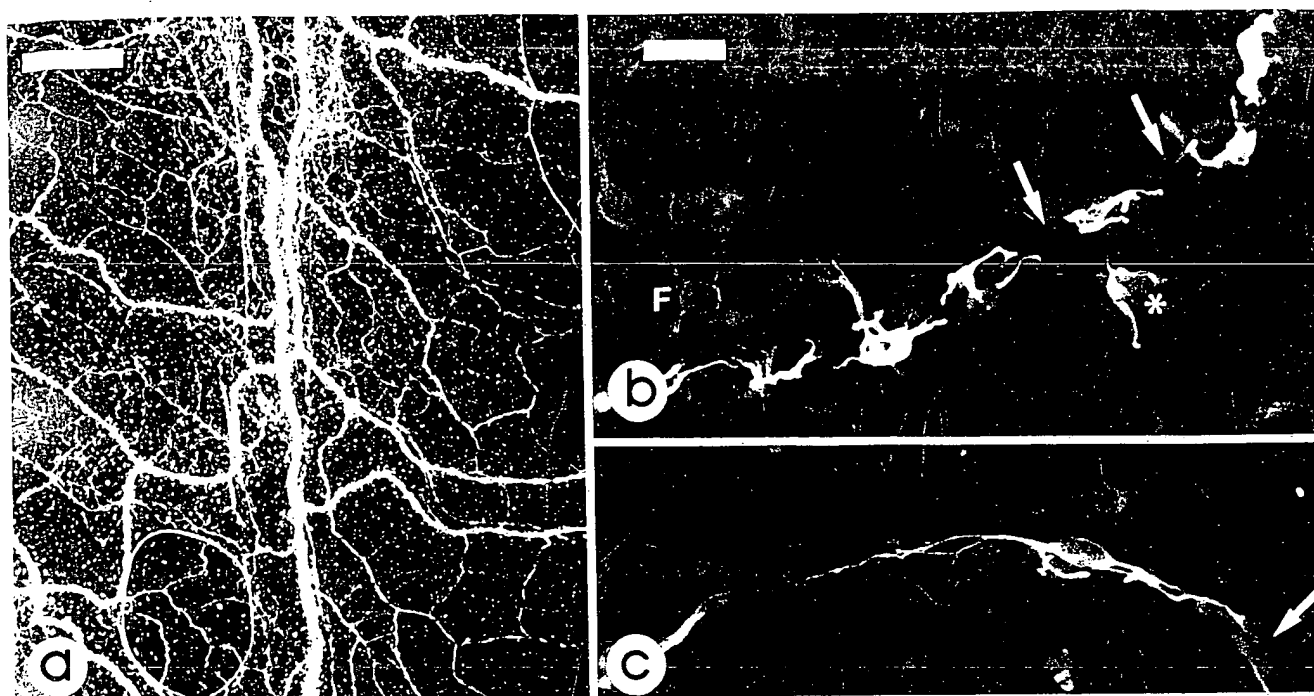


Fig. 1. a. Low-magnification view of a TRITC-phalloidin-stained area of the rat mesentery of a rat 7 days after mast cell stimulation with compound 48/80. Note bright staining of the entire capillary network. Numerous mononucleated cells (probably macrophages) are also stained. Bar: 200 μ m. b, c Visualization of pericytes in the rat mesentery by anti-desmin seven days after induction of mast cell-stimulated angiogenesis. Fibroblasts (F) and endothelial cells (arrows) remain largely unstained. In b pericytes of a postca-

pillary are shown. Note that cellular processes of postcapillary pericytes encircle endothelial cells perpendicularly to the long axis of the vessel. One fibroblast-like cell close to the vessel wall shows moderate staining for desmin and thus possibly is in the process of being transformed into a pericyte (asterisk). In c the long and slender cellular extensions of a midcapillary pericyte are shown to be aligned in parallel to the direction of the capillary. Bar: 10 μ m

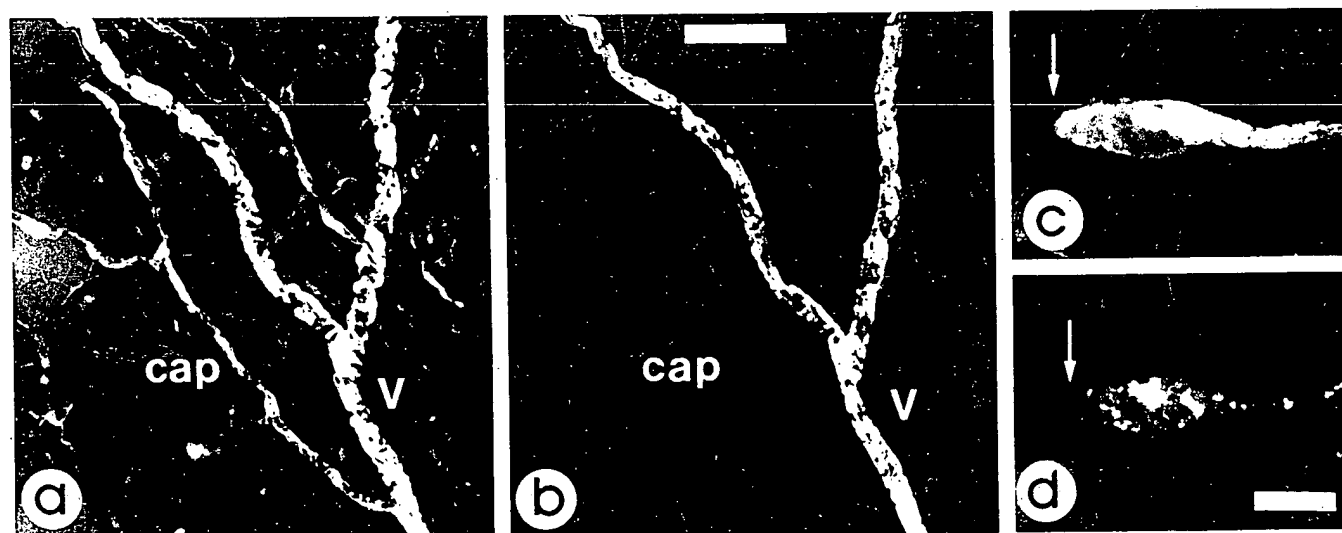


Fig. 2. a, b. Mast cell-stimulated angiogenesis in the rat mesentery. Double-immunostaining of the developing microvasculature by anti-desmin (a) and anti-SM α -actin (b). Pericytes of all microvascular segments (V venule; cap midcapillaries) are positive for desmin. The label for SM α -actin is confined to pericytes of the venule. Pericytes of midcapillaries (cap) and capillary sprouts do not contain detectable amounts of SM α -actin. Bar: 30 μ m. c, d Simulta-

neous fluorescent staining of a capillary sprout by TRITC-phalloidin (c) and an antibody to human von Willebrand factor (d). Note bright phalloidin labeling of entire endothelial cells including the growing tips. Von Willebrand factor, in contrast, is restricted to cytoplasmic granules (probably Weibel-Palade bodies) of more proximal sprout segments, leaving the leading edge (arrow) unstained. Bar: 5 μ m

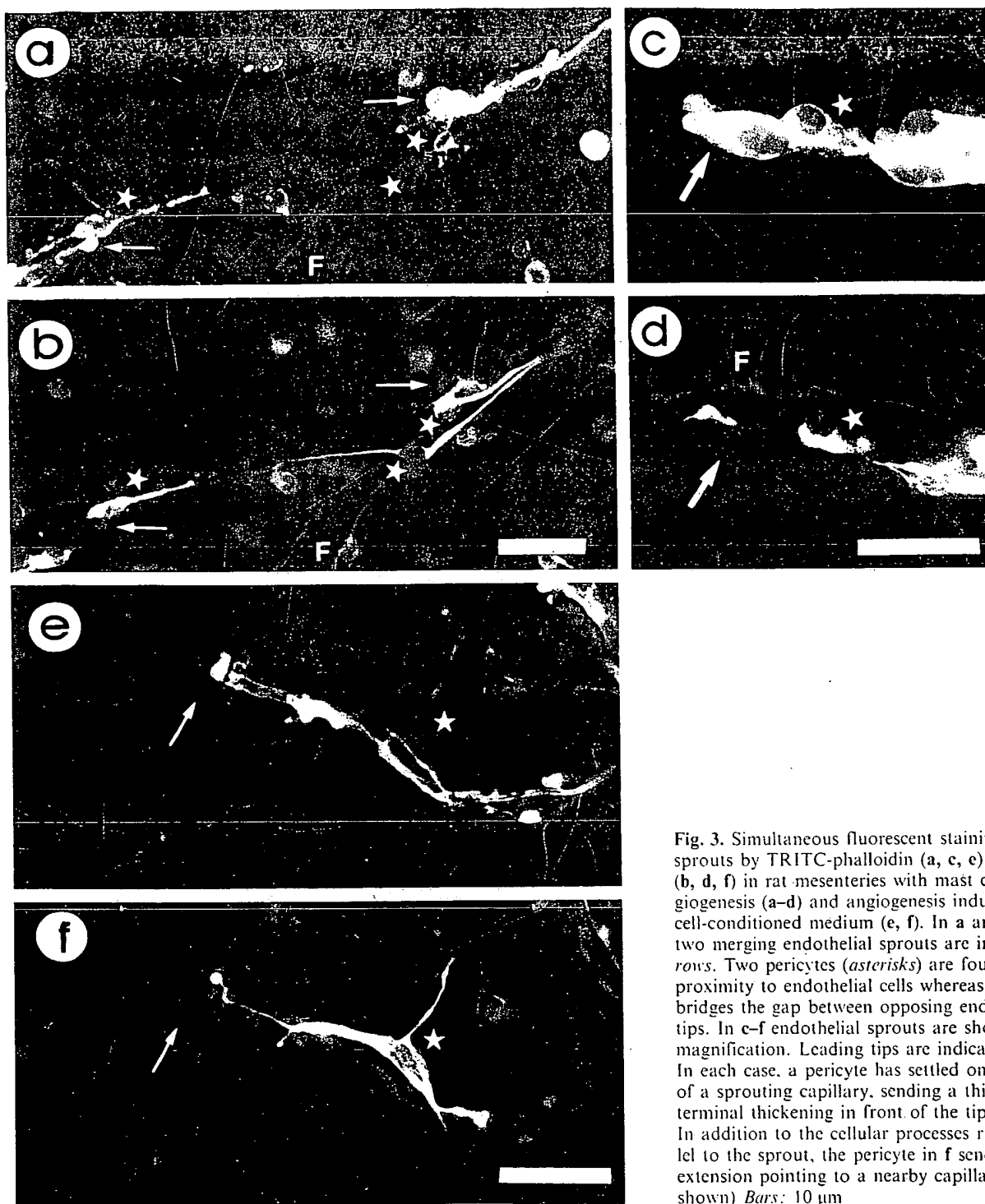


Fig. 3. Simultaneous fluorescent staining of capillary sprouts by TRITC-phalloidin (a, c, e) and anti-desmin (b, d, f) in rat mesenteries with mast cell-induced angiogenesis (a-d) and angiogenesis induced by tumor cell-conditioned medium (e, f). In a and b the tips of two merging endothelial sprouts are indicated by arrows. Two pericytes (asterisks) are found in close proximity to endothelial cells whereas a third pericyte bridges the gap between opposing endothelial sprout tips. In c-f endothelial sprouts are shown at higher magnification. Leading tips are indicated by arrows. In each case, a pericyte has settled on the tip region of a sprouting capillary, sending a thin process with a terminal thickening in front of the tip of the sprout. In addition to the cellular processes running in parallel to the sprout, the pericyte in f sends out a third extension pointing to a nearby capillary loop (not shown). Bars: 10 μ m.

mode of angiogenesis induction, i.e., by mast cell stimulation (Fig. 3a-d) or stimulation by tumor cell-conditioned medium (Fig. 3e, f). Developing pericytes were clearly reactive for desmin, thus being easily distinguishable from fibroblasts and endothelial cells. The proximity between nascent pericytes and endothelial sprouts appeared to depend on the degree of maturation of the growing capillaries. Some pericytes were seen in tight apposition to endothelial cells, thus resembling mature

pericytes, whereas other pericytes were only loosely associated with endothelial sprouts.

Nascent pericytes usually exhibited a slender, bipolar morphology with cellular processes advancing at the tips of endothelial sprouts for various distances (up to 100 μ m; Fig. 3a, b). Most interestingly, the gaps between the tips of opposing endothelial sprouts, which apparently were preparing to merge, were frequently seen to be bridged by desmin-positive pericytes (Fig. 3a, b).

Occasionally, stromal cells were detectable in the vicinity of capillary sprouts, which displayed moderate to strong staining for desmin and which showed a fibroblast-like morphology (Fig. 1b). These cells might be regarded as transitional cell types between desmin-negative fibroblasts and desmin-positive pericytes. That pericytes apparently do not develop from pre- and post-capillary pericytes or from smooth muscle cells can also be suggested from the observation that pericytes of growing capillaries were always negative for SM α -actin (Fig. 2a, b). According to these findings, immature pericytes appeared to be more closely related to stromal fibroblasts than to vascular smooth muscle cells.

Discussion

In this report we present observations that may help to explain two aspects of pericyte biology. The first aspect is concerned with the question of the developmental origin of microvascular pericytes. There is a long-standing controversy about whether pericytes originate from vascular smooth muscle cells or stromal fibroblasts (Sims 1986; Diaz-Flores et al. 1991; Crocker et al. 1970; Rhodin and Fujita 1989; Nakayasu 1988). The demonstration of certain smooth-muscle type proteins, e.g., SM α -actin (Herman and D'Amore 1985; Skalli et al. 1989) cGMP-kinase (Joyce et al. 1984), myosin (Joyce et al. 1985) and desmin (Fujimoto and Singer 1987) in microvascular pericytes has directed some attention toward the vascular smooth-muscle cell as a possible precursor cell of pericytes. However, we have recently shown that pericytes of true capillaries (midcapillaries), which may be regarded as the youngest pericytes of a capillary network, are virtually devoid of SM α -actin (Nehls and Drenckhahn 1991a). Additionally, the present study demonstrates that developing pericytes do not express detectable amounts of SM α -actin. Transitional cell types between fibroblasts and pericytes have been visualized which were fibroblast-like by morphology and locations (loose connections to sprouts) and pericyte-like by positive staining for desmin. We thus strongly support a model according to which pericytes at least partly differentiate from stromal fibroblasts. Fibroblasts are supposed to acquire pericytic characteristics in the following sequence: In a first step, cell-cell contacts between endothelial sprouts and fibroblasts are established (Rhodin and Fujita 1989). Thereafter these periendothelial cells may start expression of desmin. Finally, when microvascular networks expand to form pre- and postcapillary segments pericytes may acquire smooth muscle-like features and begin to express SM α -actin.

The second aspect of pericyte function addressed by our findings is the open question of the involvement of pericytes in capillary sprouting. An early role of pericytes in sprouting has been denied by many researchers (Paweletz and Knierim 1989; Blood and Zetter 1990; Folkman and Klagsbrun 1987; Folkman and Haudenschild 1980). In a recently published review article, for example, Blood and Zetter (1990) have drawn the following conclusions: "Formation of a basement mem-

brane and investment of capillaries with pericytes are generally associated with the end of the proliferative stage and the beginning of the mature or quiescent stage of capillary function". This and similar statements of other authors refer to ultrastructural examinations of capillary sprouts during wound healing (Crocker et al. 1970). However, the only reliable criterion that allows to distinguish stromal fibroblasts from pericytes by conventional electron microscopy is the capillary basal lamina which surrounds pericytes but not fibroblasts. Unfortunately, there is very little basal lamina material at the leading tips of capillary sprouts (Rhodin and Fujita 1989; Bär and Wolff 1972; Paku and Paweletz 1991). Thus, electron-microscope investigations do not allow to exclude the possibility that extramural pericytes are involved in capillary sprouting, which, by definition (Ashton and DeOliveira 1966), are not yet surrounded by basal lamina material. To circumvent these technical limitations which appear to hinder the investigation of pericyte development at the electron-microscope level, we and others (Verhoeven and Buysens 1988; Schlingemann et al. 1991) have chosen an immunohistochemical approach for positive identification of pericytes.

There are two more reports speaking in favor of an early involvement of pericytes in angiogenesis (Verhoeven and Buysens 1988; Schlingemann et al. 1991). However, these conclusions were based on examination of tissue sections which do not allow to follow cellular extensions for longer distances and to identify the growing tips of sprouts safely. In the present study we have used whole-mount preparations and obtained by this technique convincing data demonstrating that capillary sprouting includes coordinated growth of both pericytes and endothelial cells rather than mere elongation of endothelial tubes, which subsequently become occupied by microvascular pericytes. Moreover, the gaps between opposing endothelial sprouts, which apparently were about to merge, were regularly seen to be bridged by desmin-positive pericytes. Thus, guiding of endothelial sprouts by pericytes would offer an attractive explanation for the hitherto unsolved problem of how capillary sprouts find each other to fuse to continuous capillary loops.

Under in-vitro conditions pericytes have been shown to inhibit the proliferation of cultured endothelial cells (Orlidge and D'Amore 1987). This inhibitory effect of pericytes appeared to be mediated by an activated form of transforming growth factor β (Antonelli-Orlidge et al. 1989). Furthermore, the activation of TGF β has been shown to depend on the formation of cell-cell contacts between pericytes and endothelial cells (Antonelli-Orlidge et al. 1989). Wakui (1988) has demonstrated that the number of cell-cell contacts of endothelial cells and pericytes increases with ongoing maturation of newly formed capillaries. In view of these findings and considerations it appears reasonable to assume that stimulation and inhibition of endothelial proliferation during angiogenesis is a finely balanced process in which pericytes, by an active involvement in capillary sprouting, are likely to play a key role.

References

- Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA (1989) An activated form of transforming growth factor β is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci USA* 86:4544-4548
- Ashton A, DeOliveira F (1966) Nomenclature of pericytes. *Intra-mural and extramural*. *Br J Ophthalmol* 50:119-123
- Bär Th, Wolff JR (1972) The formation of capillary basement membranes during internal vascularization of the rat's cerebral cortex. *Z Zellforsch* 133:231-248
- Blood CH, Zetter BR (1990) Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochem Biophys Acta* 1032:89-118
- Crocker DJ, Murad TM, Geer JC (1970) Role of the pericyte in wound healing. An ultrastructural study. *Exp Mol Pathol* 13:51-65
- Díaz-Flores L, Gutiérrez R, Varela H, Rancel N, Valladares F (1991) Microvascular pericytes: a review of their morphological and functional characteristics. *Histol Histopathol* 6:269-286
- Drenckhahn D, Franz H (1986) Identification of actin, α -actinin, and vinculin-containing plaques at the lateral membrane of epithelial cells. *J Cell Biol* 102:1843-1852
- Drenckhahn D, Frotscher M, Kaiser HW (1984) Concentration of F-actin in synaptic formations of the hippocampus as visualized by staining with fluorescent phalloidin. *Brain Res* 300:381-384
- Folkman J, Haudenschild C (1980) Angiogenesis in vitro. *Nature* 288:551-556
- Folkman J, Klagsbrun M (1987) Angiogenic factors. *Science* 235:442-447
- Fujimoto T, Singer SJ (1987) Immunocytochemical studies of desmin and vimentin in pericapillary cells of chicken. *J Histochem Cytochem* 35:1105-1115
- Herman IM, D'Amore PA (1985) Microvascular pericytes contain muscle and non-muscle actins. *J Cell Biol* 101:43-52
- Joyce NC, DeCamilli P, Boyles J (1984) Pericytes, like vascular smooth muscle cells, are immunocytochemically positive for cyclic GMP-dependent protein kinase. *Microvasc Res* 28:206-219
- Joyce NC, Haire MF, Palade GE (1985) Contractile proteins in pericytes. II. Immunocytochemical evidence for the presence of two isomyosins in graded concentrations. *J Cell Biol* 100:1387-1395
- Nakayasu K (1988) Origin of pericytes in neovascularization of rat cornea. *Jpn J Ophthalmol* 32:105-112
- Nehls V, Drenckhahn D (1991a) Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J Cell Biol* 113:147-154
- Nehls V, Drenckhahn D (1991b) Demonstration of actin filament stress fibers in microvascular endothelial cells in situ. *Microvasc Res* 42:103-112
- Norrby K, Jakobsson A, Sörbo J (1986) Mast cell-mediated angiogenesis: a novel experimental model using the rat mesentery. *Virchows Arch [B]* 52:195-206
- Orlidge A, D'Amore PA (1987) Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 105:1455-1462
- Paku S, Paweletz N (1991) First steps of tumor-related angiogenesis. *Lab Invest* 65:334-346
- Paweletz N, Knierim M (1989) Tumor-related angiogenesis. *Crit Rev Oncol/Hematol* 9:197-242
- Pepper MS, Belin D, Montesano R, Orci L, Vassalli J-D (1990) Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J Cell Biol* 111:743-755
- Rhodin JAG, Fujita H (1989) Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. *J Submicrosc Cytol Pathol* 21:1-34
- Sappino AP, Schürch W, Gabbiani G (1990) Biology of disease. Differentiation repertoire of fibroblastic cells: Expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab Invest* 63:144-161
- Schlingemann RO, Rietveld FJR, Kwaspen F, Kerkhof PCM van de, Waal RMW de, Ruiter DJ (1991) Differential expression of markers for endothelial cells, pericytes, and basal lamina in the microvasculature of tumors and granulation tissue. *Am J Pathol* 138:1335-1347
- Sims DE (1986) The pericyte - a review. *Tissue Cell* 18:153-174
- Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillesse D, Gabbiani G (1986) A mAb against alpha-smooth muscle actin: A new probe for smooth muscle differentiation. *J Cell Biol* 103:2787-2796
- Skalli O, Pelte M-F, Peclet M-C, Gabbiani G, Gugliotta P, Bussolati G, Ravazzola M, Orci L (1989) Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J Histochem Cytochem* 37:315-321
- Verhoeven D, Buysse N (1988) Desmin-positive stellate cells associated with angiogenesis in a tumour and non-tumour system. *Virchows Arch [B]* 54:263-272
- Wakui S (1988) Two- and three-dimensional ultrastructural observation of two cell angiogenesis in human granulation tissue. *Virch Arch [B]* 56:127-139

Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses

G. RHODIN and H. FUJITA

Department of Anatomy, College of Medicine, University of South Florida, Tampa, Florida 33612, USA,
and Department of Anatomy, Osaka University Medical School, Osaka, Japan

SUMMARY - Capillary sprouting was studied by a combination of intravital video recording and subsequent electron microscopy in the mesentery of young female rats without previous experimental manipulation. Selected segments of the mesenteric microvascular bed with capillary sprouts were carefully surveyed and mapped at a monitor magnification of 255 times and submitted to detailed *in vivo* analysis concerning flow pattern and cells at 2000 times magnification. The mesentery was preserved for light and electron microscopy by a superfusion of glutaraldehyde while observed and recorded on video, confirming earlier investigations that this type of fixation does preserve exceptionally well vascular topography and diameters of the mesenteric microvascular bed. Capillary sprouts originated as endothelial spurs from arteriolar-venular arcades and continued to grow in size through a bipolar rearrangement of endothelial cells, forming a solid sprout tip which progressively lengthened by alternately rapid and slow growth phases. The extended leading tip of the migrating endothelial cells displayed microspikes and pseudopodia denuded of basal lamina. The cytoplasm of the leading tip contained an array of microtubules, 75 Å filaments and many small vesicles. In similarity with the situation in nerve growth cones, all these organelles probably participate in cytoplasmic streaming and cell migration. The sprout lumen arose between endothelial cells of the solid sprout. Mesenteric connective tissue fibroblasts approached and settled down on the sprouts, being converted to pericytes as the fibroblasts became enveloped by a basal lamina. The pericytes reinforced the wall of the delicate and fragile capillary sprouts, and trapped plasma, platelets and red blood cells which had leaked out temporarily, in the process assuming an umbrella shape. The arteriolar feeder of the arcades was surrounded by cells which were classified as intermediate between pericytes and true smooth muscle cells. Sprouts that presumably were on the verge of merging with other capillaries were analyzed for indications of how anastomoses are formed.

KEY WORDS - microcirculation - angiogenesis - capillary growth - rat mesentery - intravital - electron microscopy

INTRODUCTION

The structural mechanism of capillary growth is fairly well understood. It is a process which begins with a focal degradation of the capillary basal lamina and the projection of endothelial processes from the parent vessel into the surrounding connective tissue. Gradually, several en-

dothelial cells become polarized and protrude in an amoeboid migratory movement, establishing a solid capillary sprout which elongates through endothelial mitotic activity several cells behind the leading tip. A narrow sprout lumen emerges between component endothelial cells as blood plasma and formed elements of the blood gradually enter the sprout. Anastomosis of sprouts and adjacent capillaries leads to the onset of blood flow. Much of what is known is based on studies of vascular growth in wounds in the cornea (Schoeffl, 1963; Sholley *et al.*, 1984) and cremaster muscle (Schoeffl, 1963) after cauterization; growth of vessels in the ear chamber implanted in the rabbit ear (Cliff, 1963, 1965); and *in vitro* investigations

This paper was presented in part at the Fourth World Congress for Microcirculation, Tokyo, Japan, July 26-30, 1987.

of cultured endothelial cells (Folkman and Haudenschild, 1980). The pioneering studies of capillary growth in the tail fins of tadpoles (Clark, 1912, 1918; Clark and Clark, 1925) and the rabbit ear chamber (Clark and Clark, 1934, 1939, 1940, 1943; Clark *et al.*, 1930) contributed considerably to the exploration of the basic developmental pattern of microvessels in both amphibians and mammals by utilizing a camera lucida technique.

However, the normal growth and development of capillaries without previous experimental intervention has not been studied in a mammalian system. The mesentery of young rats does offer such a system, since a microvascular bed is a prerequisite for subsequent deposition of lipids and the development of adipose tissue. This investigation combined intravital video recordings with light and electron microscope analyses of the same microvascular segment. The video play backs enabled us to study in detail the flow patterns of arteriolar-venular arcades and the oscillating plasma columns in the capillary sprouts which originated from these arcades. One disadvantage of our technical approach is the inability to follow the actual growth of capillary sprouts by time-lapse video recording over prolonged periods, since the rat mesentery can not be exposed and observed for more than 1-2 h without causing damage, although light, to the microvascular bed, and it is technically difficult to expose and observe the mesentery more than once. However, the strength of our approach is the ability to analyze by electron microscopy every vascular segment and every cell recorded by video, and to ascertain by serial sectioning that these components are explored in their entirety, thereby avoiding misinterpretations which often arise as a result of analyzing single sections by transmission electron microscopy.

This investigation confirmed many of the observations made by previous investigators who utilized an experimental model, thus establishing the similarity between normal vascular growth and one produced experimentally. Our findings, dealing with the assumed migration of endothelial cells during the growth process, brought to light the close structural similarity between this system and those of nerve growth cones, fibroblast movements, and the activity of reticulopods of protozoans, since the same organelles are present and the same bipolarity of cells exist. Furthermore, a certain developmental pattern was confirmed to exist, since it was demonstrated that fibroblasts do transform to capillary pericytes, and that the pericytes in turn represent precursors of smooth muscle cells as arterioles and venules emerge from their capillary precursors.

MATERIALS AND METHODS

Experimental animals - A total of 24 female 3-4 week-old Sprague-Dawley or Wistar-Kyoto rats, weighing between 100-130 grams were used.

Anesthesia - The rats were given an intra-abdominal injection of sodium pentobarbital, 50 mg/kg anesthetic dose of 60 mg/ml sodium. At the end of the experiment, each rat was killed by an intra-venous injection of air.

Intravital microscopy - The microscope used for *in vivo* observations was a slightly modified Leitz Orthoplan light microscope, equipped with a large stage for a plastic animal cradle; two long-working distance Leitz objectives (5× with 0.10 N.A. and 25× with 0.22 N.A.); a Sony color television camera connected to a monitor; a 3/4" U-matic color video recorder, and a Panasonic time/date generator Model WJ-800 for superimposition of time and date onto the video signal. The magnifications obtained on the TV monitor were 255× with the 5× objective and 2,000× with the 25× objective. The field was observed and recorded from the moment the rat was put on the microscope stage until the end of the fixation. First, the entire field was surveyed with the 5× objective in order to establish and record the main flow patterns, and to find areas with capillary sprouts. Once the proper segment of the microvascular bed was identified, the subsequent recordings were done with the 25× objective.

Fixation - The mesenteric membranes of all rats were fixed by superfusion fixation. Through a mid-line abdominal incision, a short part of the small intestine with its mesentery was exteriorized and put into a plastic cradle, designed so that the mesentery could be constantly bathed in a saline solution. The mesenteric membrane was held down flat and slightly stretched out by a brass ring. A slow, continuous stream of 37 °C saline solution was kept moving across the mesentery from inside the ring during observation of the field. This stream was exchanged for a gentle flooding of fixation solution at the onset of the fixation process.

Solutions - For fixation at 37 °C, a solution of 2% glutaraldehyde in 0.1 mol/liter cacodylate buffer was used as the initial fixative for a period of 15 min. Then followed immediately fixation with a 1% osmium tetroxide solution in 0.1 mol/liter cacodylate buffer at room temperature for another 15 min. Physiological saline solution at 37 °C was continuously superfused across the mesenteric membrane during the pre-fixation period of observation, which would last from 60 to 120 min.

Dehydration - After fixation with glutaraldehyde and osmium tetroxide, the entire area of mesentery inside the brass ring was flooded with 70% ethanol for 60 min. The mesenteric 'window' covered by the brass ring was then excised, transferred to a Petri dish with 70% ethanol and pinned down to a piece of dental wax, using fine needles, taking care to keep the mesenteric membrane flat and moderately taut. The pinned-down preparation was then dehydrated in a graded series of ethanols, approximately 30 min in each concentration.

Embedding - After 30 min in 100% ethanol, the pins and dental wax were removed, and the mesenteric membrane was submerged in propylene oxide, and subsequently in 50/50 mixture of propylene oxide and Epon 812. The specimen membrane was then transferred to a peel-away flat mold and pinned down. Great care was taken to keep the membrane flat, stretched, unwrinkled and without trapping air bubbles under the mesentery. Epon 812 at 100% was added and the preparation allowed to polymerize.

Sectioning - The mesenteric membrane was sectioned in a plane parallel to its flat surface. The mesenteric microvascular bed is arranged essentially in a two-dimensional pattern. Selected areas were sectioned either parallel to or perpendicularly to the long axes of the microvessels and the capillary sprouts. Thick (1 µm) or thin (50-100 nm) serial sections were cut on a MT2B Ultratome, using Diatome diamond knives. The thick sections were stained with toluidine blue for light microscopy.

Ribbons of thin serial sections were mounted on formvar-coated slot grids and stained with uranyl acetate followed by lead citrate.

Light microscopy - The embedded mesenteric membrane was photographed before sectioning in a whole-mounted preparation at magnifications of 100 \times and 400 \times . Selected frames on the television monitor were also photographed and compared with those obtained after Epon embedding and sectioning. Photographs were also taken of serially sectioned vascular segments, mounted on glass slides and stained with toluidine blue. These were quite suitable for analysis of vascular components with 100 \times oil immersion objective.

Electron microscopy - Thin sections were examined with a Philips 301 transmission electron microscope at plate magnifications ranging between 250 \times and 35,000 \times . The electron microscope analyses identified each specific segment of the microvascular bed and established cell types and components of the vascular walls, including endothelial cells, pericytes, smooth muscle cells and perivascular connective tissue elements, guided by earlier investigations by Rhodin (1967, 1968, 1972, 1974, 1980, 1984, 1986).

INTRAVITAL OBSERVATIONS

Vascular components

The microvascular bed of the young rat mesentery was formed by a two-dimensional network of arterioles, capillaries and venules (Figs. 1, 10 and 47). These microvessels continued to develop and grow in young and adult rats in advance of lipid deposition and the development of adipose tissue. The outer edges of the developing microvascular beds displayed arteriolar-venular arcades and loops from which emanated capillary sprouts. These sprouts averaged 5 μ m in diameter and extended anywhere from 5 μ m to 1000 μ m. Most of the capillary sprouts originated at angular points, where the microvessel changed from an arteriole to a venule, although sprouts arose also at other points, in which case twice as many emanated from venous segments as opposed to arterial segments.

The direction of the blood flow was easily seen and recorded during the observation of the microvascular bed in the intravital microscope. The flow was rapid in the arteriole. These microvessels underwent periodic constrictions and dilatations, a phenomenon referred to as vasomotion. There was no margination of polymorphonuclear leukocytes in arterioles. Venules were classified as such segments of the microvascular bed, based on a less rapid blood flow, compared to the arterioles, and on the fact that the leukocytes were often margined in these vessels. The margination was observed as round or drop-shaped highly refractile objects sliding and rolling along the venular margins.

Most capillary sprouts contained occasional erythrocytes

and platelets which were observed to gain access to the narrow lumen near the base of origin of the sprout. These formed elements of the blood were suspended in plasma columns, and oscillating pulsations were observed in the sprout lumen in synchronization with the heart beats. The tips of the sprouts were very delicate, averaging 2 μ m in diameter (Fig. 12), or they were quite blunt and as wide as the base of the sprout, averaging 6 μ m (Fig. 24). The pointed tips usually did not contain a lumen. They were extremely thin and blended with the surrounding collagenous and elastic fibers. In fact, it was often impossible to tell the difference between an elastic fiber and the extended, delicate tip of a capillary sprout (Fig. 12). In the sprouts with a blunt leading end, a lumen was present also near the bulbous end. Other sprouts were extremely short, shaped as a spur, and were often discovered only after fixation and embedding of the mesenteric membrane (Figs. 3 and 4). However, once found in the whole-mounted preparation under the light microscope, they could also be detected in the video play-back of that particular region of the mesenteric microvascular bed. Sprouts were found which formed short loops, probably heading toward a merger with other sprouts and capillaries, thus establishing an anastomosis (Fig. 50). In these loops, erythrocytes and platelets could be present.

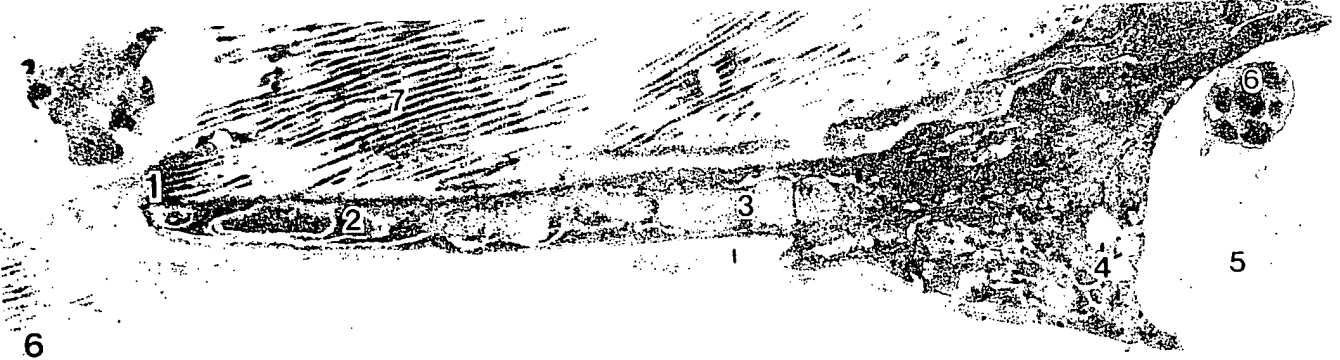
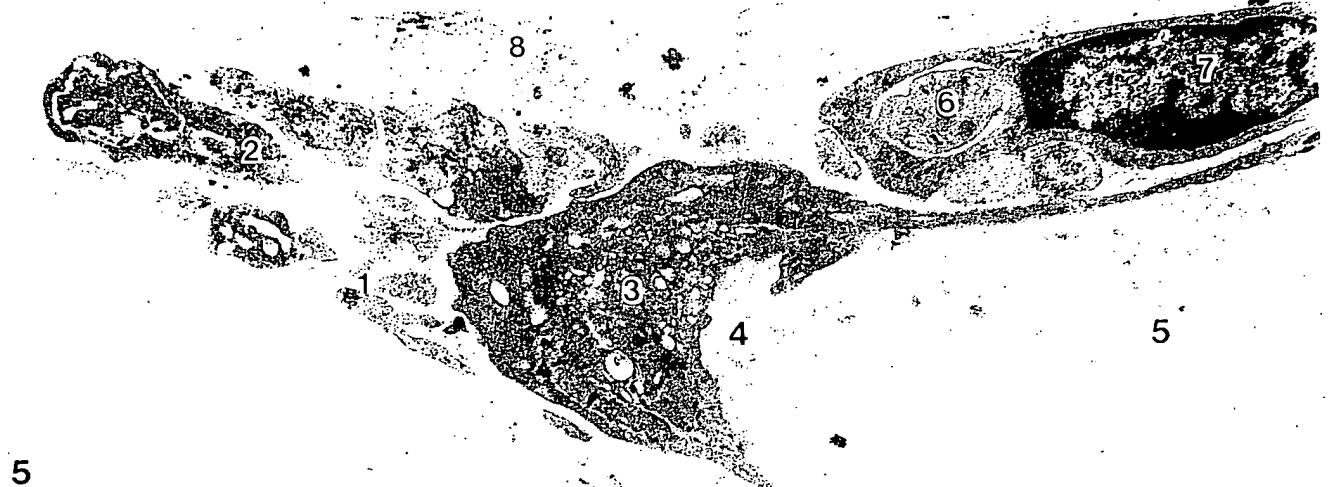
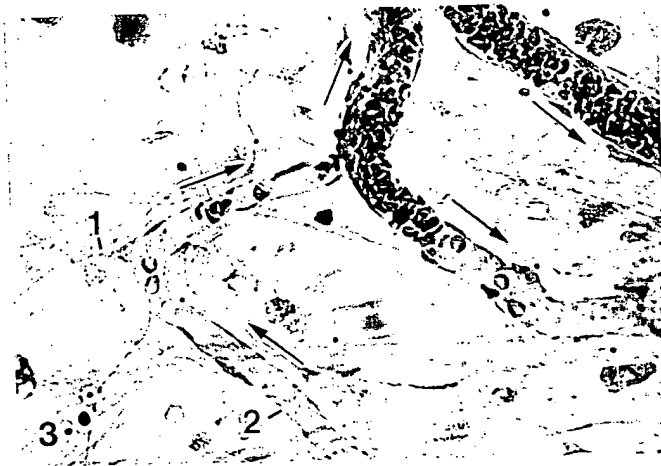
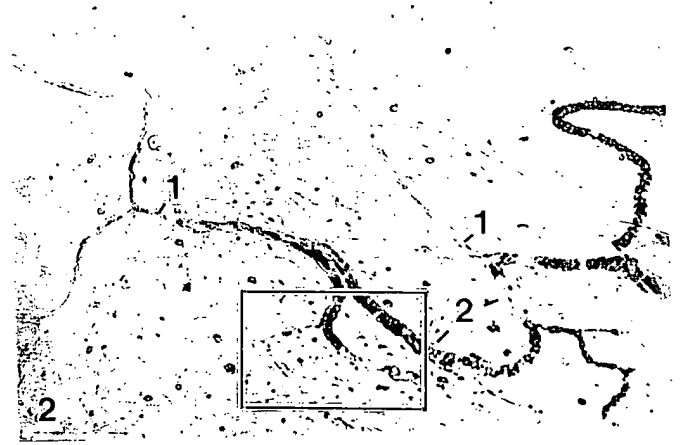
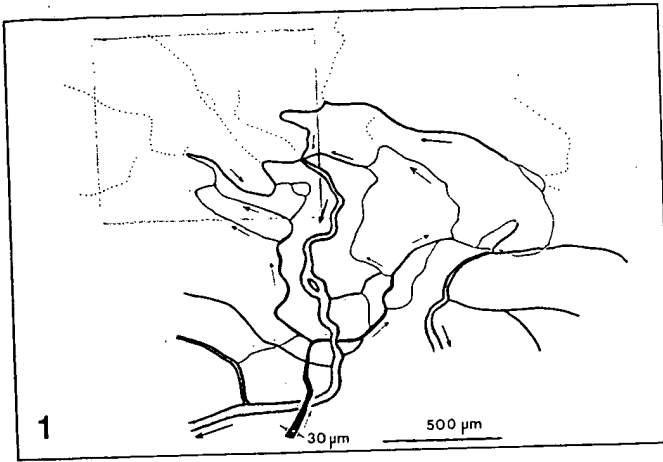
The variation in sprout lengths and topographic relationship to other perivascular elements of the mesenteric membrane obviously reflected stages of sprouting and capillary growth, which were further explored by light and electron microscope analyses.

Perivascular elements

Pericytes were identified as periendothelial cells, closely associated with capillaries and capillary sprouts (Fig. 12). Their nuclei were highly heterochromatic, and thus easily observed in the living preparation, emerging as small bumps on the connective tissue aspect of the capillary sprouts.

Mast cells were frequently found in close association with the leading tips of capillary sprouts (Fig. 41). They varied in shape, some oval or oblong but the majority were round. As a rule, the degranulated mast cells were not as easily detected as those which had not undergone degranulation.

Fibroblasts and **macrophages** were present in the connective tissue space of the mesenteric membrane. However, these cells were generally not easily detected in the intravital microscope but emerged after fixation and embedding, and their location could then be pinpointed in video play-backs.



LIGHT AND ELECTRON MICROSCOPE OBSERVATIONS

There was a positive identification of each microvascular segment analyzed in the light and the electron microscopes, since the entire mesenteric window, recorded on video, was subsequently studied in the whole-mounted Epon embedded preparation, photographed and compared with the intravital video recording. Selected segments of the microvessels were isolated and sectioned serially, either parallel to or at right angle to the longitudinal axis of the vessel. It was, therefore, easy to analyze a specific arteriolar-venular loop or a capillary sprout by step-wise increasing the magnification from the light microscope level to low, medium and high magnifications in the electron microscope (Figs. 1 to 6).

The shape, width and length of the capillary sprouts analyzed varied from the smallest and most narrow extensions in the endothelial cells of the arteriolar-venular loops to very long and slender sprouts extending for distances of up to 1000 μm . The description below follows an assumed path of development and growth of the capillary sprouts. It is recognized that the video recordings of the sprouts did not last for more than two hours at the

most and that a recording of actual growth did not take place. This will be further reviewed in the discussion of our findings. The description deals with the following, carefully chosen stages of an assumed sprout developmental pattern: endothelial spurs, short sprouts, long sprouts, and vascular anastomoses.

Endothelial spurs

The first indications of spur formation were small cytoplasmic pseudopodic extensions of the endothelial cells which penetrated the basal lamina of the vascular segment from which they emerged (Fig. 9). The endothelial pseudopodia could be short and narrow as well as long and bulbous. They contained a slightly flocculent but otherwise structureless cytoplasm devoid of cell organelles, except a small number of ribosomes and several layers of delicate membranes, assumed to represent smooth endoplasmic reticulum (Fig. 8). At the time the extensions took on the shape of a spur (Fig. 5), they might contain mitochondria and profiles of rough endoplasmic reticulum. Even the nucleus of the endothelial cell of their origin was seen to slide into the spur (Fig. 9).

FIGURE 1 Tracing of an intravital video recording of a rat mesentery microvascular bed supplied by one 30 μm arteriole and drained by two venules. The arteriole gives rise to arteriolar (red)-venular (blue) arcades from which emanate several capillary sprouts (dotted yellow-green). All blue microvascular segments showed leukocyte margination, the red segments none. Arrows indicate blood flow direction. Figs. 2 to 6 form a continuation enlargement of a selected part of this tracing (green rectangle), identifying the origin and structure of an endothelial spur.

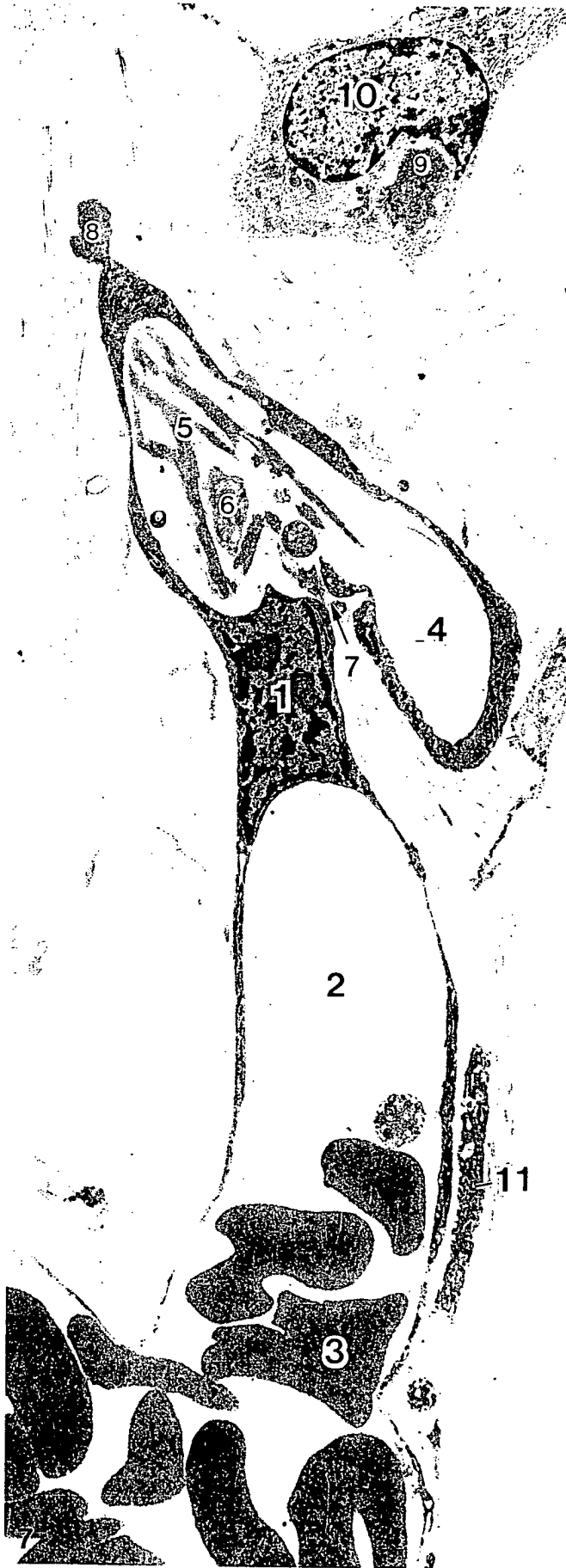
FIGURE 2 Microvessels present within green rectangle in Fig. 1 seen in a light micrograph of a fixed and Epon embedded whole-mount before sectioning. Two long capillary sprouts (1) take their origins at points where the arterial-venous arcades (2) make sharp angular turns. $\times 100$. 1 mm = 1 mm.

FIGURE 3 Enlargement of rectangle in Fig. 2 in a whole-mount preparation showing a small endothelial spur (1) protruding from an arterial feeder (2) of an arcade. The spur was first observed after fixation and embedding and was barely seen in video play-backs of this region. Arrows indicate blood flow direction. $\times 400$. 1 mm = 2.5 μm .

FIGURE 4 Electron micrograph of arterial feeder with the endothelial spur (1) seen in Fig. 3. The microvessel has the structural characteristics of a capillary with thin endothelial cytoplasm (2), and an occasional pericyte (3). A fibroblast (4) and a macrophage (5) are close to the microvessel, and two erythrocytes (6) remain in its lumen. Arrow indicates blood flow direction. $\times 2,700$. 1 mm = 0.37 μm .

FIGURE 5 Enlargement of endothelial spur seen in Fig. 4. The plane of sectioning is slightly off center of spur, confirmed by serial sections. The spur consists of filamentous (1) as well as bulbous (2) endothelial extensions, denuded of basal lamina. A highly vesicular Golgi area (3) is seen. A small lumen (4) represents an out-pocketing from the arterial capillary lumen (5). An endothelial extension (6) is engulfed by the pericyte (7). The connective tissue interstitium shows delicate collagenous fibrils (8). $\times 12,000$. 1 mm = 0.078 μm = 78 nm.

FIGURE 6 Central section of the endothelial spur seen in Fig. 5. This consists of a single solid but slender endothelial extension with a leading tip (1). Its cytoplasm displays profiles of rough endoplasmic reticulum (2), mitochondria (3), and 7.5 nm filaments (not resolved at this magnification). Golgi vacuoles (4) are seen near the spur lumen (5) with a platelet (6). Note the change in orientation of collagenous fibrils (7) compared to Fig. 5. $\times 11,200$. 1 mm = 0.089 μm = 89 nm.



Often, the spurs contained a highly vesicular Golgi region. In addition, nests of highly electron-dense whirls of smooth membranes were present, a cytoplasmic structure reminiscent of so-called myelin figures. Once the extensions took on the shape of an endothelial spur, a thin basal lamina was present on the connective tissue aspect of the spur. In the adjacent connective tissue, several cells were usually present. They were mast cells, macrophages and fibroblasts (Fig. 4). Of these cells, the fibroblast was always found in very close association with the endothelial spur, often enclosing one or several of the endothelial extensions. The topographic relationships between the fibroblasts and the extensions of the endothelial cells could take many shapes but usually represented a peg-and-socket arrangement without special junctional arrangements between opposing cell membranes (Fig. 8). Since these fibroblasts often displayed an incomplete basal lamina, they could be considered pericytes. This is further explored below in relation to transformation of fibroblasts into pericytes.

Short sprouts

There were short projections from an arteriolar-venular loop and obviously varied in length (Fig. 7). The main difference between an endothelial spur and a short sprout is the fact that the spur is usually formed by cytoplasmic

extensions from only one endothelial cell, whereas the short sprouts showed the participation of several endothelial cells, surrounding a small but distinct lumen. The nucleus of each participating endothelial cell became elongated and flattened as the endothelial spur was transformed into a short sprout. Infrequently, mitotic figures were recorded in the short sprouts. However, in most sprouts studied, it was the elongated (Fig. 26) or irregularly shaped (Fig. 45) endothelial nucleus which dominated the picture. The thin endothelial cytoplasm contained centrioles, ribosomes, and many profiles of rough endoplasmic reticulum. The leading end of the short sprouts varied in its configuration, probably depending on the speed of the migration of the endothelial cells. The end could be blunt (Fig. 41), or it could be frayed (Fig. 55). Sprouts with frayed ends displayed elongations of the endothelial cytoplasm and discontinuities whereby the sprout lumen was in direct continuity with the extracellular matrix of the connective tissue compartment, demonstrated by serial sections (Figs. 54 and 55). In such instances, platelets and erythrocytes had escaped from the sprout lumen, and there was fibrin deposition. In fact, there often seemed to be a matrix space or channel present into which blood elements and plasma escaped, and where the fibrin was precipitated. These 'channels' were at times delineated by a thin basal lamina, marking the border toward the connective tissue matrix (Fig. 55).

FIGURE 7 Electron micrograph of a short capillary sprout (marked '4' in Fig. 11), analyzed in its entirety in a long series of thin sections. It is made up of two endothelial cells, the nucleus (1) of one seen in this selected section at mid-level of the sprout. The sprout lumen (2) near the sprout base contains erythrocytes (3), and is connected with the lumen (4) at the sprout leading end, containing fibrinoid (5) and platelets (6). The endothelial cytoplasm is thin and shows one discontinuity (7) at this level. One bulbous endothelial extension (8) forms the leading tip and another extension (9) is engulfed by a nearby fibroblast (10). A fibroblast/pericyte (11) is seen near the sprout base. $\times 5,600$. 1 mm = 0.18 μ m.

FIGURE 8 Enlargement of fibroblast in Fig. 7. The fibroblast cytoplasm (1), rich in rough endoplasmic reticulum (2), completely surrounds one bulbous extension (3) of the capillary sprout. The overall cytoplasmic density of the endothelial extensions is greater than that of the surrounding fibroblast due to a greater density of monoribosomes. Delicate intracellular membranes (4) probably represent smooth endoplasmic reticulum. A mitochondrion is present (5). There are no junctional specializations between fibroblast and endothelial cell membranes seen in this section. $\times 35,700$. 1 mm = 0.028 μ m = 28 nm.

FIGURE 9 Electron micrograph of the initial appearance of endothelial extensions in an arterial feeder of a capillary loop (1). Bulbous endothelial projections (2) with a structureless, fluffy cytoplasm pierce the basal lamina and continue to penetrate the interstitial connective tissue (3). At some point, the nucleus (4) of the parent endothelial cell enters the bulbous projection in a motion reminiscent of the extravasation of a polymorphonuclear leukocyte. Myelin figures (5) are present in the projection. Segment of a pericyte (6) and collagen fibers (7) are seen. Arrow indicates direction of blood flow. This is a section selected from a long sequence of serial sections of this microvascular segment. $\times 19,000$. 1 mm = 0.053 μ m = 53 nm.

One or several fibroblasts were always situated near the short sprouts (Fig. 25). Some surrounded the base of the sprout, assuming the shape and location of pericytes. Others were near the leading tip of the sprout. In either case, there were cellular contacts between the endothelial cell and the fibroblast in the typical peg-and-socket arrangement (Fig. 31).

Long sprouts

The continuous growth of capillary sprouts is known to occur through mitotic divisions of endothelial cells with subsequent migration of the endothelial cells and lengthening of the sprout. Our structural analysis of the present material identified two types of long capillary sprouts: those with long pointed, delicate tips, and those with blunt leading ends. As will be discussed later in this communication, it is assumed that the two types represent different stages of capillary growth, but they will be described separately.

Long pointed sprouts - The leading end of this type of

capillary sprout usually consisted of an extremely long and delicate extension of a single endothelial cell, with a slightly club-shaped tip provided with many microspikes, filopodia and bulbous excrescencies (Fig. 14). As a rule, the leading tip and its cytoplasmic processes were bare and not covered by a basal lamina. However, just proximally to the leading tip, a basal lamina invariably enveloped the narrow endothelial sprout. The cytoplasm of the leading sprout tip was characterized by a small number of ribosomes, mitochondria and small cytoplasmic vesicles, the latter averaging 700 Å in diameter. There were also numerous filaments averaging 75 Å in diameter and some microtubules averaging 250 Å in diameter (Figs. 18 and 21). The bulbous excrescencies were generally structureless, except for a flocculent cytoplasmic ground substance and a few ribosomes. They were similar in structure to those seen as initial signs of the capillary sprouting, described above under 'endothelial spurs'. The narrow microspikes contained only very delicate filaments, averaging less than 50 Å in diameter.

The mid-portion of the long capillary sprouts usually

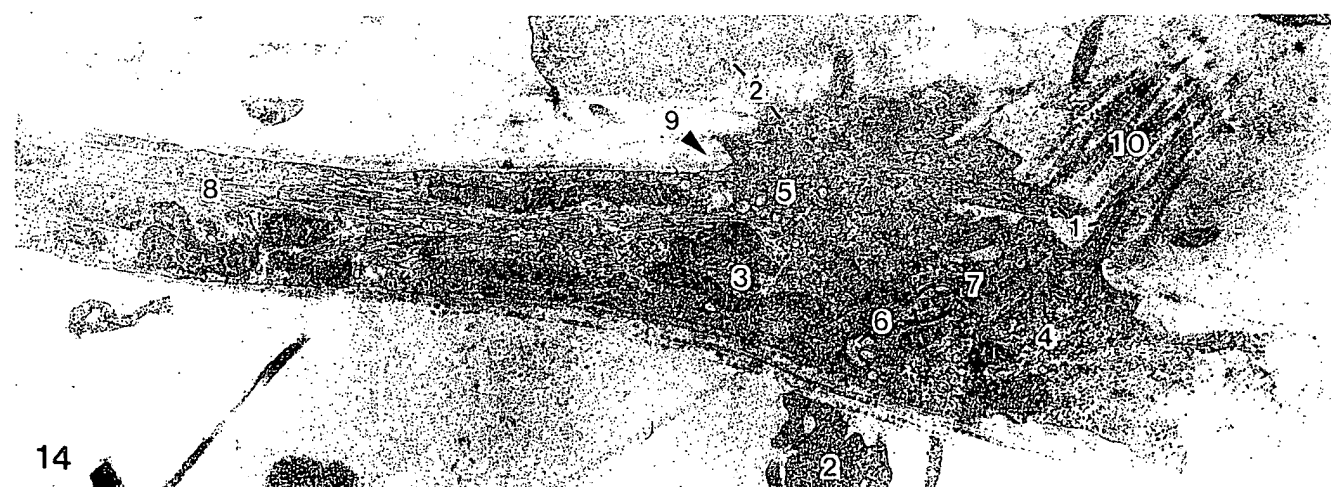
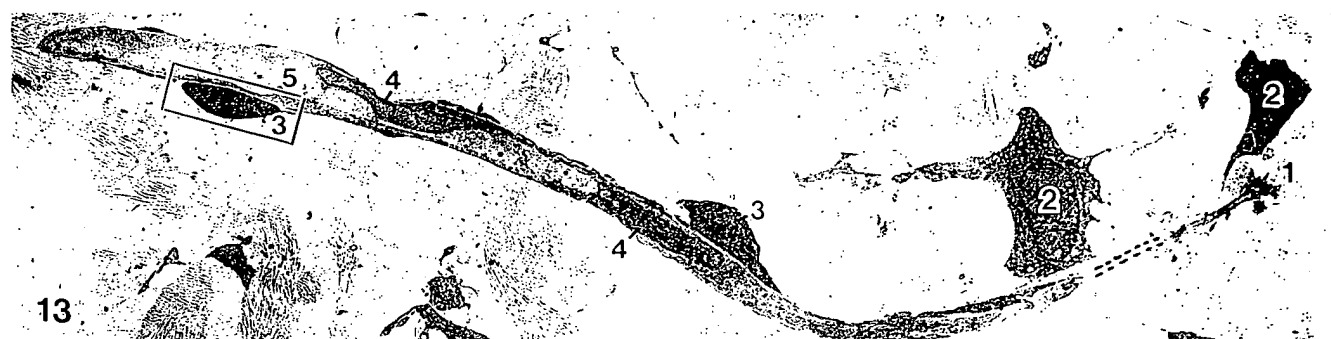
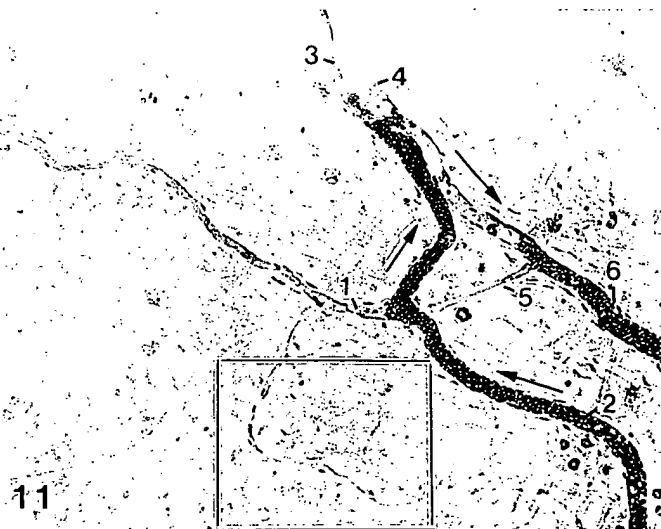
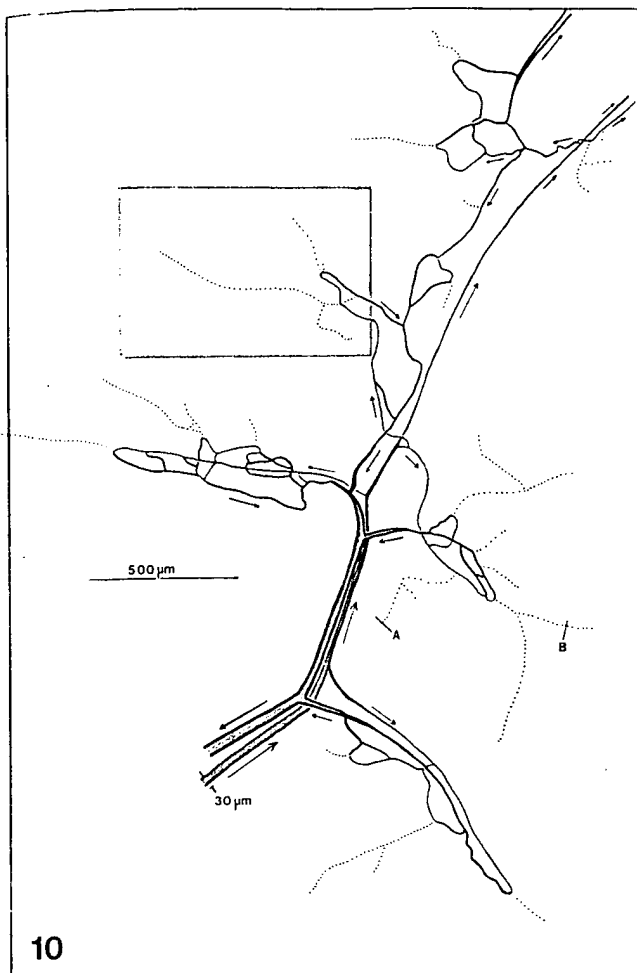
FIGURE 10 Tracing of a typical microvascular area of a 4 week-old rat mesentery. The flow direction in arterioles and arterial capillaries (red) as well as in venules and venous capillaries (blue) was confirmed by intravital video recording. Capillary sprouts (dotted yellow-green) showed oscillating plasma columns, and originated mostly at angular points where the microvascular loop changed from an arterial to a venous segment. This was confirmed by the presence of marginated leukocytes in the venous part of the microvessels and the absence thereof in the arterial part. Arrows indicate blood flow direction. Figs. 11 to 14 form a continuum of enlargements of a selected part of this tracing (green rectangle) identifying the origin and structure of a long, pointed capillary sprout. Sprouts marked 'A' and 'B' are analyzed in cross sections in Figs. 19 and 20.

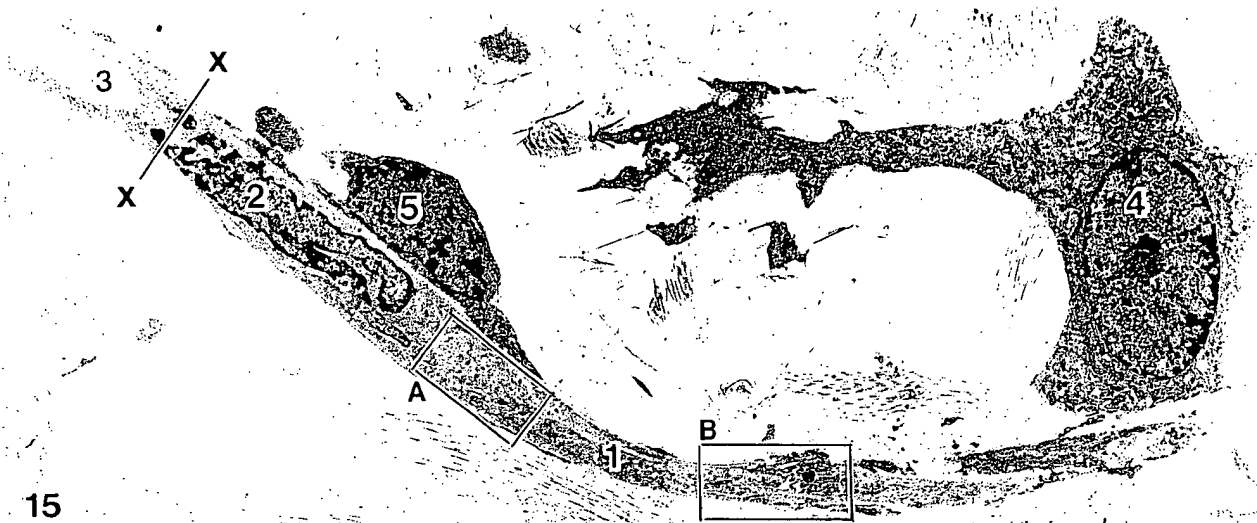
FIGURE 11 Microvessels present within green rectangle in Fig. 10 seen in a light micrograph of a fixed and Epon embedded whole-mount before sectioning. This demonstrates a typical arteriolar-venular arcade in the mesentery of a 4 week-old rat. One branching capillary sprout (1) develops from the arterial feeder (2) of the loop, one long sprout (3) and one short (4) (enlarged in Fig. 7) arise from the angular point of the loop, and yet another sprout (5) (enlarged in Fig. 41) takes its origin from the venous drainer (6) of the loop. Arrows indicate blood flow direction. $\times 126$. 1 mm = 7.9 μ m.

FIGURE 12 Enlargement of rectangle in Fig. 11 showing a long, curving pointed capillary sprout expanding among bundles of collagenous and elastic fibers of the mesenteric connective tissue. Some erythrocytes (1) are present in the narrow lumen of the proximal part of the sprout, and pericytes (2) are attached to the distal leading part of the sprout. $\times 435$. 1 mm = 2.3 μ m.

FIGURE 13 Electron micrograph of the distal leading end of the long pointed sprout in Fig. 12 demonstrating the most peripheral tip (1), two fibroblasts (2), two pericytes (3), two endothelial nuclei (4) and a primitive sprout lumen (5). The two dashed lines represent the continuity of the leading tip, confirmed by serial sections. Pericyte in rectangle is enlarged in Fig. 33. Details of other parts of this sprout are seen in Figs. 14, 15 to 18, 29, 30 and 32. $\times 18,300$. 1 mm = 0.055 μ m = 55 nm.

FIGURE 14 Enlargement of the leading tip of a long pointed sprout. This is one of many serial sections through this sprout tip, but not the same section as in Fig. 13. There are several microspikes (1) with microfilaments at the tip, as well as bulbous projections (2) with a flocculent, rather structureless content. There is ample representation of cell organelles in the leading tip of the capillary sprout, including mitochondria (3), free ribosomes (4), small vesicles (5), some large vacuoles (6), smooth membranes (7) and 7.5 nm filaments (8). The delicate basal lamina (9) is discontinued at the very tip, thus leaving the microprojections devoid of this extracellular component and in direct contact with reticular and collagen fibers (10). Figs. 10 to 14 demonstrate the superiority of the technical approach used in this investigation. The identical microvascular segment, recorded *in vivo*, can be analyzed by increasingly larger magnifications from light microscopy to electron microscopy. $\times 18,300$. 1 mm = 0.055 μ m = 55 nm.





consisted of a single long and narrow extension of a single endothelial cell, but the extension of a second endothelial cell was also encountered at this level. This was demonstrated positively by serial sections parallel to the long axis of the sprout (Figs. 13 and 15), as well as by serial sections of sprouts perpendicular to their long axes and taken at various levels of the long pointed sprouts (Figs. 19 and 20). The cytoplasm of these extensions contained numerous 250 Å microtubules, bundles of 75 Å filaments, many 700 Å vesicles, ribosomes and polysomes as well as short profiles of rough endoplasmic reticulum, mitochondria, multivesicular bodies, and occasional secondary lysosomes (Fig. 17). The Golgi area was populated mostly by vesicular components, some small and some large (Fig. 16). From the centrally located centrioles extended 250 Å microtubules which continued distally in the sprout (Fig. 16). The nuclei of the endothelial cells which formed the capillary sprout were elongated with a slightly uneven surface (Fig. 15). They averaged 25 µm in length, and their cross-sectioned profile was oval or half-moon-shaped with a maximum diameter of 5 µm and a minimum diameter of 0.5 µm (Fig. 20). Mitotic figures were not recorded in the endothelial cells at this level of the sprouts in the present investigative material. The proximal part of the long capillary sprouts invariably displayed a primitive lumen, and there were more than two endothelial cells present (Figs. 13, 15 and 20). The lumen of the sprout first appeared as a slit-like separation

of two endothelial cell extensions, held together by poorly differentiated junctional areas of opposing cell membranes with adjacent cytoplasm occupied by bundles of densely packed microfilaments (Figs. 20 and 21). More proximally, the lumen became wider and one encountered platelets and occasional erythrocytes, as well as chylomicra and the flocculent, finely granular ('fluffy') material which is typical for fixed and electron stained blood plasma. There were no other elements present in this primitive lumen, except in rare cases (Fig. 53), fibrin or fibrinoid (Movat and More, 1957) sometimes in crystalline form (Hüttner *et al.*, 1968). Polymorphonuclear leukocytes and lymphocytes were not seen in capillary sprouts. The cytoplasm of the endothelial cells which made up the lining of the proximal part of the long capillary sprouts had all the characteristics typical of mature endothelial cells of connective tissue capillaries. Occasionally, mitotic figures were recorded in the endothelial cells located in the proximal part of the long sprouts.

Long blunt sprouts - The leading end of this type of capillary sprout was quite blunt, surrounded by several fibroblasts in close apposition (Figs. 24 to 26). Its width was equal to that of the rest of the sprout, averaging 6 µm. Another characteristic feature was the sprout lumen, which was wide open throughout the sprout from its origin all the way to the distal end, and often contained a single row of closely packed erythrocytes alternating with

FIGURE 15 Enlargement of the leading end of the long pointed capillary sprout seen in Fig. 13 in yet another serial section. It is formed by a solid cytoplasmic process (1) from one elongated, bipolar, endothelial cell with its nucleus (2). A narrow process of a second endothelial cell approaches from behind, seen in cross section in Fig. 20. The line x-x marks the level of that cross section. A sprout lumen (3) develops gradually when focal separations of apposing endothelial cells merge. A neighboring fibroblast (4) approaches the sprout and settles down on the sprout surface, in the process being transformed into a pericyte (5). $\times 2,900$. 1 mm = 0.34 µm.

FIGURE 16 Enlargement of the rectangle A in Fig. 15, analyzed in another serial section. It shows details of the centriolar and Golgi regions of an endothelial cell during the process of extension and migration of a capillary sprout. Microtubules (1) extend from the centriole (2). The Golgi region (3) contains a large number of vesicular components, some large (4) and many small (5), some of which are of the coated variety. The peripheral regions of this area of the endothelial process display 7.5 nm filaments (6), ribosomes (7), rough endoplasmic reticulum (8) and plasmalemmal vesicles (9) most of which are detached from the cell membrane. $\times 45,000$. 1 mm = 0.022 µm = 22 nm = 220 Å.

FIGURE 17 Enlargement of rectangle B in Fig. 15, analyzed in the same serial section as Fig. 16. In this particular region of the bipolar endothelial cell, free ribosomes (1) and small profiles of rough endoplasmic reticulum (2) are present throughout the endothelial process. It can be assumed that mitochondria (3), vesicles (4) and occasional lysosomes (5) track along the delicate bundles of 7.5 nm filaments (6) and microtubules (not seen in this micrograph) in a process of cytoplasmic streaming during capillary sprout migration. $\times 35,000$. 1 mm = 0.029 µm = 29 nm = 290 Å.

long columns of loosely packed platelets. Fibrinoid was also present in limited amounts. At least two endothelial cells participated in the formation of the blunt leading end. Their large and irregularly shaped nuclei were situated near the leading ends of the cells (Fig. 28).

The endothelial cytoplasm of the blunt ends contained a very large number of closely packed and irregularly arranged 75 Å filaments, numerous ribosomes and a few, very small mitochondria. Junctional areas of adjacent endothelial cells were poorly differentiated. The most distal end of the endothelial cells often displayed small bulbous cytoplasmic extensions which penetrated the delicate basal lamina of the leading blunt tip (Fig. 27). These extensions contained only ribosomes. They also made contact with neighboring fibroblasts in a peg-and-socket arrangement (Fig. 31). It is believed that the long blunt sprouts represented a slow phase of capillary growth during which the endothelial cells prepared for a more rapid phase of endothelial migration, and perhaps mitosis, which resulted in the long pointed sprouts. This theory will be further elaborated in the discussion.

Pericytes

By definition, pericytes are highly branched perivascular cells which are closely associated with capillaries and postcapillary venules. They are attached to the connective tissue aspect of the endothelial cells, and their branched cytoplasm surrounds the microvessel. In association with capillaries, they occur singly at certain intervals. Postcapillary venules usually have a higher number of pericytes, which may form a complete layer of cells in venules of larger caliber. A pericyte must be surrounded by a basal lamina in order to qualify as such. Ordinarily, there are several junctional areas between pericytes and endothelial cells, some classified as tight junctions, others as gap junctions (Tilton *et al.*, 1979; Sims, 1986).

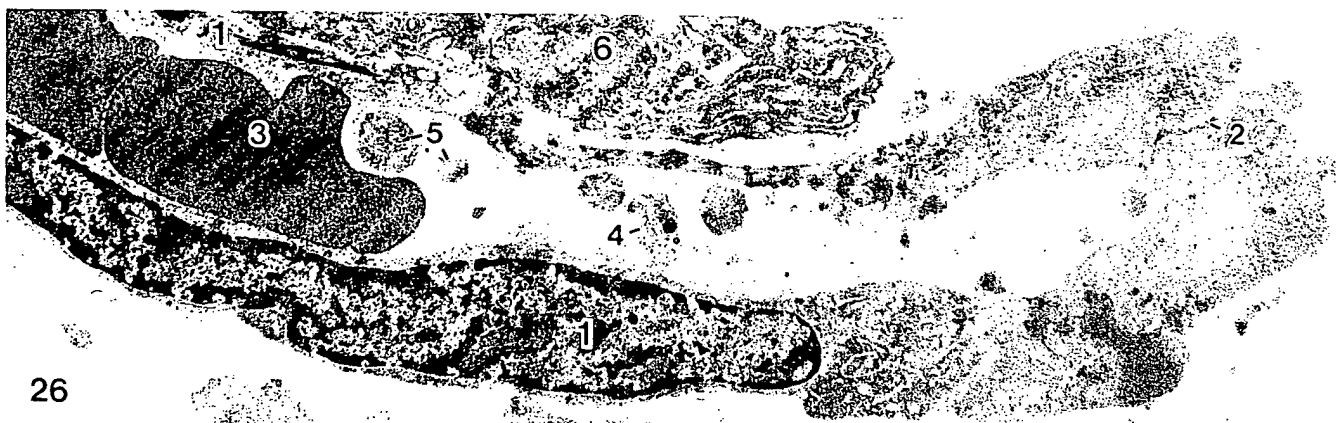
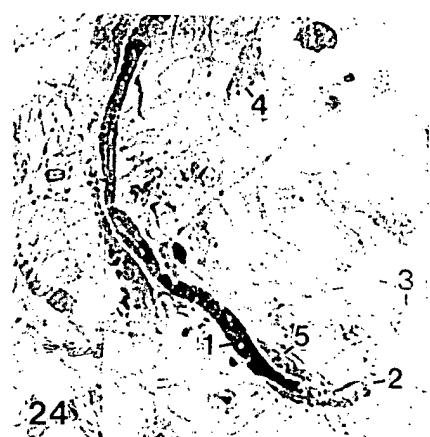
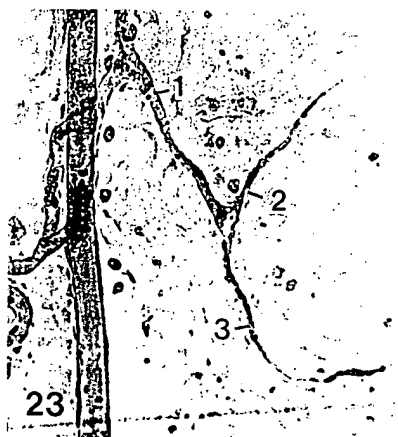
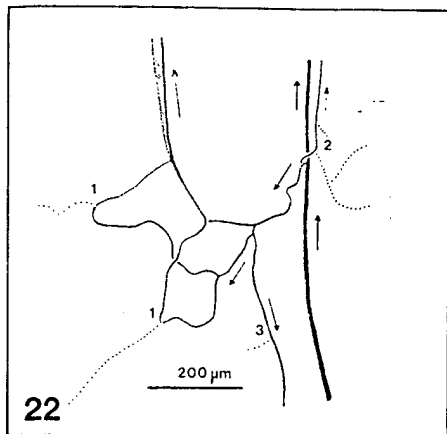
Cells which fit this description were present regularly in association with most sprouts examined in this investigation (Figs. 15 and 33). They were less often observed to be associated with endothelial spurs and short sprouts. However, they were, as a rule, present in association with

FIGURE 18 Detail of the longitudinally sectioned distal, single extension of the bipolar endothelial cell seen in Fig. 14. This part is just behind the leading tip of the extension. Its cytoplasm contains mitochondria (1), small cytoplasmic vesicles (2), ribosomes (3) and many 7.5 nm filaments (4). It is enveloped by a delicate basal lamina (5) and reticular filaments (6). A short filopodium (7) projects laterally. It is not covered by a basal lamina and its cytoplasm is slightly denser than the parent endothelial extension. $\times 45,000$. $1 \text{ mm} = 0.022 \mu\text{m} = 22 \text{ nm} = 220 \text{ \AA}$.

FIGURE 19 Cross section of the distal, single extension of a bipolar endothelial cell at approximately the same level seen in Fig. 18. The endothelial extension contains a few ribosomes (1), one mitochondrion (2), two vacuoles (3), small cytoplasmic vesicles (4) and microtubules (arrowheads). Short filopodia (6) project laterally across the delicate basal lamina (7). An elastic fiber (8), elastic microfilaments (9) and collagen fibrils (10) establish a scaffold for the migration of the endothelial extension. Cytoplasmic extensions (11) of approaching fibroblasts are also present. This is a cross section of the capillary sprout marked 'A' in Fig. 10. The leading tip was identified in video play-backs and by light microscopy after fixation and embedding before serial sectioning for electron microscopy. $\times 45,000$. $1 \text{ mm} = 0.022 \mu\text{m} = 22 \text{ nm} = 220 \text{ \AA}$.

FIGURE 20 Cross section of a long pointed capillary sprout at approximately the level indicated by a line x-x in Fig. 15. Two endothelial extensions are present. The leading extension is sectioned at the level of the endothelial nucleus (1), the trailing extension (2) is smaller. The intercellular space is opened up slightly, the beginning of a sprout lumen (3) containing flocculent and fibrinoid material (4). Primitive cell junctions occur peripherally (5). The capillary sprout tracks along an elastic fiber (6). This is a cross section of the capillary sprout marked 'B' in Fig. 10. The leading tip was identified in video play-backs and by light microscopy after fixation and embedding before serial sectioning for electron microscopy. The level of this cross section is about 50 μm proximally from the leading tip. $\times 33,000$. $1 \text{ mm} = 0.03 \mu\text{m} = 30 \text{ nm}$.

FIGURE 21 Enlargement of an area corresponding to the rectangle in Fig. 20 in a serial section close to the one in Fig. 20. The sprout lumen (1) arises through a gradual dilatation of the space between the two endothelial cells, but lateral regions remain in close apposition, displaying the appearance of primitive cell junctions (2) with adjacent dense cytoplasm containing delicate microfilaments. Other regions of the endothelial cytoplasm contain ribosomes (3), cytoplasmic vesicles (4), vacuoles (5), 7.5 nm filaments (6), microtubules (arrowheads), and mitochondria (7). The basal lamina (8) is delicate next to the endothelial cells but does not envelop the narrow extension of an adjacent fibroblast. $\times 47,000$. $1 \text{ mm} = 0.021 \mu\text{m} = 21 \text{ nm} = 210 \text{ \AA}$.



long sprouts, and then located either at mid-level or near the proximal base of a sprout. The nucleus usually had an irregular surface. The cytoplasm was more electron-dense than adjacent endothelial cell cytoplasm. There were many intermediate filaments and microtubules present, among a small number of mitochondria and profiles of rough endoplasmic reticulum. The Golgi area was extremely small, and only occasionally were there lysosomes present. Cytoplasmic vesicles were sparse.

Connective tissue elements associated with sprouts

Fibers - All capillary sprouts and their leading ends were surrounded by bundles of delicate collagenous fibrils as well as the filamentous components of elastic fibers, averaging 100 Å in diameter (Figs. 19 and 20). There was also a large elastic fiber, consisting of the amorphous component and the associated delicate filaments which formed a scaffold or supporting framework along which the migration of the capillary sprout apparently took place.

Fibroblasts - Although not easily observed in the intravital microscope, there were numerous fibroblasts present in the connective tissue compartment of the mesenteric membrane, some in close proximity to the sprouts (Fig. 15). The fibroblasts were usually very flat cells with many cy-

toplasmic extensions. Their cytoplasm contained many profiles of rough endoplasmic reticulum, free ribosomes, mitochondria, a large Golgi area, centrioles, some lysosomes, and many microtubules and intermediate filaments. The cell membrane and the cytoplasm near the cell surface were provided with numerous vesicles, most of which were of the non-coated variety. The fibroblasts did not display a basal lamina on the cytoplasmic aspect of their cell membrane. The fibroblasts contained only one nucleus which usually was round. In the present material, fibroblasts were often seen to undergo cell division.

Mast cells - Many mast cells were present near capillary sprouts, although they were never seen in direct contact with endothelial cells (Figs. 41 and 51). As indicated by the intravital observations, the mast cells were particularly numerous near the leading tip of the long pointed capillary sprouts. Sometimes the mast cells were in the stage of degranulation, but in the majority of the cases they were not.

Macrophages - In similarity with fibroblasts, macrophages were difficult to identify with certainty in the intravital microscope. Based on light and electron microscope analyses of sectioned specimens, they were often located near the leading tip of capillary sprouts (Fig. 4). Occasionally, the macrophages had established membranous contacts with endothelial cells and pericytes.

FIGURE 22 Upper right area of the traced microvascular bed seen in Fig. 10, showing several capillary sprouts (dotted yellow-green) as well as arterioles (red) and venules (blue). There is no fixed pattern as to where sprouts emerge. Most often they will arise from arterial-venous loops (1) or venous capillaries (2) and less often from arterial capillaries (3).

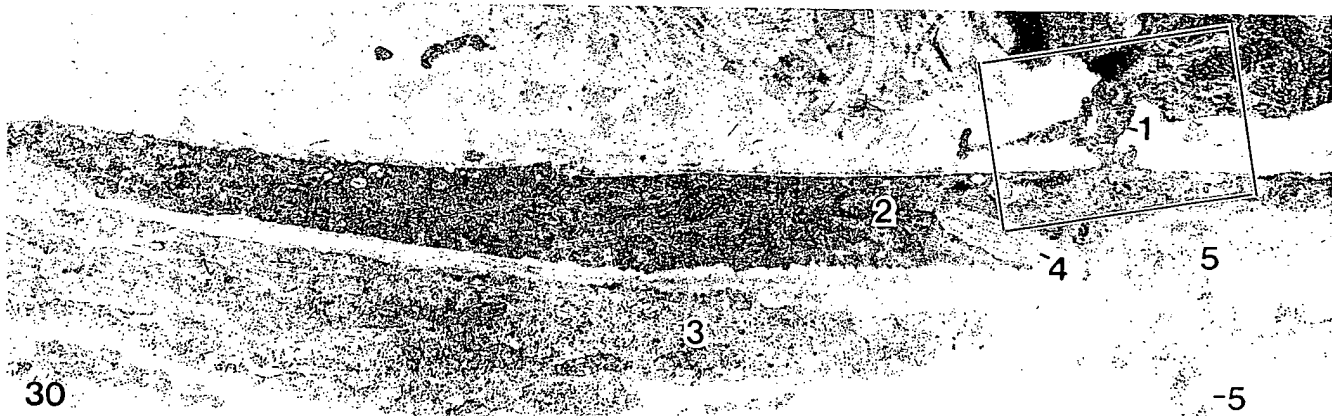
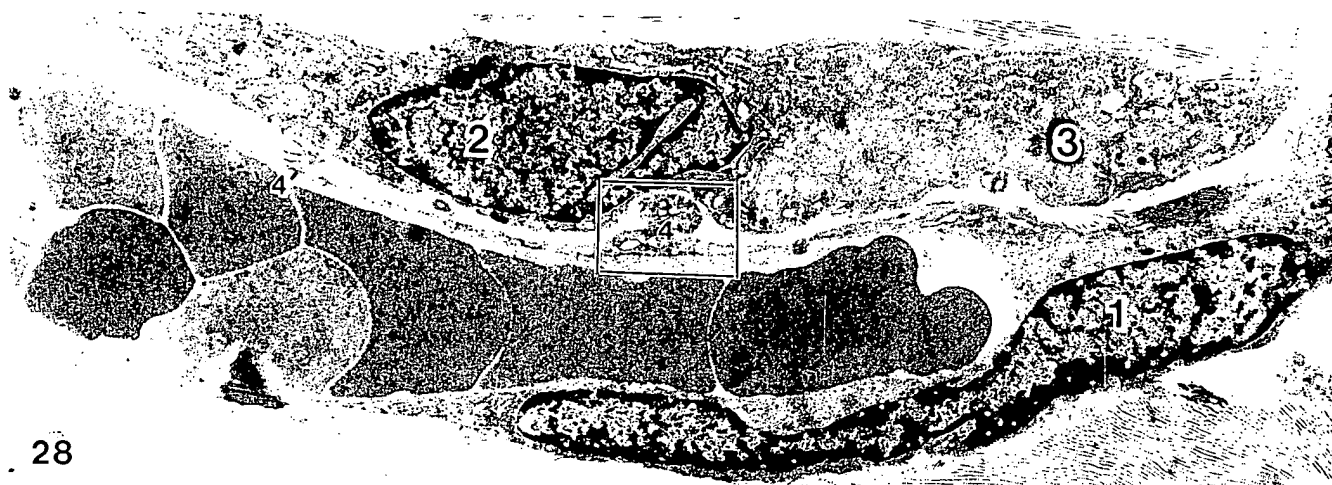
FIGURE 23 Microvessels present within green rectangle in Fig. 22 seen in a light micrograph of a fixed and Epon embedded whole-mount before sectioning. The main capillary sprout (1) comes off a venous capillary and soon gives rise to two other sprouts, one of which (2) is of the long pointed variety (similar to sprout in Fig. 12), the other (3) is of the long blunt variety. $\times 148$. 1 mm = 6.8 μ m.

FIGURE 24 Enlargement of the long blunt sprout in Fig. 23 migrating through the loose network of collagenous and elastic fibers. Erythrocytes (1) are present in the narrow lumen of the sprout almost up to the distal leading blunt end of the sprout (2). Mast cells (3) are readily recognized, whereas fibroblasts (4) and perivascular cells (5) require electron microscopy for positive identification. $\times 234$. 1 mm = 4.3 μ m.

FIGURE 25 Electron micrograph of the distal leading end of the long blunt sprout in Fig. 24. The tip (1) seems solid with an elongated endothelial nucleus (2) close by and a second endothelial nucleus (3) more proximally. An erythrocyte 'rouleaux' fills the distal part of the sprout lumen (4), trailed by densely packed platelets (5). Perivascular cells (6) are fibroblasts on the verge of settling down on the sprout, in the process transforming into pericytes. $\times 1,780$. 1 mm = 0.56 μ m.

FIGURE 26 Enlargement of the leading end of the sprout in Fig. 25. This is a different serial section from the one in Fig. 25, demonstrating that the sprout tip is not solid but contains a lumen all the way. At least two endothelial cells make up the sprout tip, judged by the two nuclei (1) and one junctional area (2). There are several small and bulbous filopodia extending from the leading endothelial surfaces. The lumen contains erythrocytes (3), a platelet (4) and small balls which are assumed to represent fibrinoid (5) material. The fibroblast (6) is subjected to further analysis in Figs. 28 and 31. $\times 5,300$. 1 mm = 0.19 μ m.

FIGURE 27 Detail of the tip of the long blunt sprout in Fig. 26 from yet a different serial section of this area. The two participating endothelial cells show a junctional region (arrowheads) which has focal densities, perhaps reflecting a transient relationship. The sprout lumen (1) contains fibrinoid material. The cytoplasm of the left endothelial cell is extremely rich in ribosomes (2), whereas the right cell cytoplasm is rich in filaments (3). The most distal endothelial surface shows several small filopodia (4) and is devoid of a basal lamina. $\times 16,000$. 1 mm = 0.063 μ m = 63 nm.



Transformation of fibroblasts to pericytes

Although the origin of pericytes, as well as their function, has not been clearly and unequivocally determined, several investigations indicate that its precursor cell is the fibroblast, and that one of the main functions very likely is its role as a precursor to smooth muscle cells. During this investigation, certain observations were made which seem to indicate that fibroblasts have, indeed, transformed into pericytes.

Fibroblasts were always present near the sprouts examined in our material (Figs. 7, 15 and 28). Mesenteric fibroblasts in general resembled fried eggs. They had a flat cytoplasm and a round nucleus. Many fibroblasts near the short as well as the long sprouts, were seen to undergo mitosis and were found in close association with the leading endothelial cells of the leading tip of the sprout, surrounding the growing capillary with their flat cytoplasm, and their nucleus becoming more elongated. Small cytoplasmic extensions from both the endothelial cells and the fibroblasts established membranous contacts

with apposing cells, at times with a peg-and-socket arrangement (Fig. 31), at other times achieving more extensive areas of contact (Fig. 30). In either case, the basal lamina of the endothelial sprout would not be present between adjacent cell membranes. In several cases, it was established through serial sections that one or several cytoplasmic extensions of the fibroblasts had settled down on the surface of the endothelial cell and had become covered by the same basal lamina that covered the endothelial cell (Fig. 30). A cord-like continuity between the cytoplasm of the fibroblast and its broad cytoplasmic extension on the surface of the endothelial cell was not surrounded by a basal lamina (Fig. 32). By definition fibroblasts do not have a basal lamina. During the transformation process, the fibroblast became separated from the connective tissue milieu by the same basal lamina that envelopes the endothelial cells of the sprout, as evidenced by the illustration in Fig. 32, where the major 'free' part of the fibroblast was not covered by a basal lamina. It can be assumed that we fortuitously captured a moment during which a fibroblast was becoming incor-

FIGURE 28 Enlargement of the leading end of the long blunt sprout seen in Fig. 25. The nucleus (1) of one of the leading endothelial cells is typically large and elongated, perhaps reflecting a state of DNA replication, making the cell polyploid. The perivascular cell (2) is a fibroblast. It is not enveloped by a basal lamina, and it has a more extensive rough endoplasmic reticulum (3) than is present in a pericyte. Several small endothelial extensions (4) make contact with the fibroblast. $\times 4,900$. $1 \text{ mm} = 0.20 \text{ }\mu\text{m}$.

FIGURE 29 Topography of the leading end of the long pointed sprout seen in Fig. 15. A fibroblast (1) has approached the leading extension (2) of the endothelial cell (3), wrapping its cytoplasm around the extension (4). The cell to the left is a pericyte (5), since its enveloping basal lamina can be resolved at higher magnifications. $\times 3,900$. $1 \text{ mm} = 0.26 \text{ }\mu\text{m}$.

FIGURE 30 Enlarged detail of the relationship between the fibroblast and the endothelial extension in Fig. 29. This is one of several serial sections of this area. At this level of sectioning, there appears a cord-like attenuation of the fibroblast cytoplasm (1) which is continuous with a flat and dense cytoplasmic wrapping (2) around the endothelial extension (3) with a less dense cytoplasm. The endothelial extension shows nodal filamentous (4) and bulbous (5) micropodia. $\times 12,000$. $1 \text{ mm} = 0.083 \text{ }\mu\text{m}$.

FIGURE 31 Enlargement of rectangular area in Fig. 28, showing the peg-and-socket relationship between an endothelial cell and a perivascular fibroblast. Both cells have a rich supply of free ribosomes (1) and profiles of rough endoplasmic reticulum (2). The area of cell membrane contact within the peg-and-socket does not show any junctional specialization, although the peg-and-socket structure was analyzed in its entirety in serial section. A faint basal lamina (3) is resolved in some areas, but is absent in relation to the fibroblast cell membrane (arrowheads) and also between cells. $\times 41,000$. $1 \text{ mm} = 0.024 \text{ }\mu\text{m} = 24 \text{ nm} = 240 \text{ }\text{\AA}$.

FIGURE 32 Enlargement of rectangular area in Fig. 30, demonstrating the cord-like continuity (1) between the cytoplasm of the fibroblast (2) and what is rightly referred to as the pericyte (3). Note the presence of a basal lamina (4) at the surface of the pericytic cytoplasm, and the absence of this structure (arrowheads) in relation to the cord and the fibroblast. The high cytoplasmic density of the pericyte wrapping (5) is mostly due to numerous intermediate filaments. $\times 45,000$. $1 \text{ mm} = 0.022 \text{ }\mu\text{m} = 22 \text{ nm} = 220 \text{ }\text{\AA}$.

porated within the wall of the sprout, and ultimately differentiated into a pericyte.

Pericytes and their changing role when capillary sprouts are transformed into capillaries, arterioles and venules

It has been suggested that pericytes serve as *modulators of leakage* from microvessels, particularly postcapillary venules (Sims, 1986). Thus, leaked proteins, tracer particles, platelets and erythrocytes are trapped under pericytes or their basal laminae. In the present investigative material, it was observed intravitaly that erythrocytes as well as margined polymorphonuclear leukocytes would often be en route across the endothelial walls of capillary sprouts as well as postcapillary venules and venous drainers (Fig. 35). Subsequent analyses of such microvascular segments, as well as others, in sections for light and electron microscopy, demonstrated that the delicate and fragile endothelial walls displayed cytoplasmic gaps, allowing the escape of plasma, platelets, erythrocytes and occasional leukocytes. Although such cytoplasmic gaps could be found anywhere along the sprouts and venous segments of the microvessels, the escaped plasma and formed elements of the blood had often accumulated under pericytes and would not, as a rule, be found outside of the basal lamina of the pericytes. In fact, these accumulations pushed the pericytes away from the microvessel, rendering the pericyte an umbrella-shape (Fig. 34). Images were

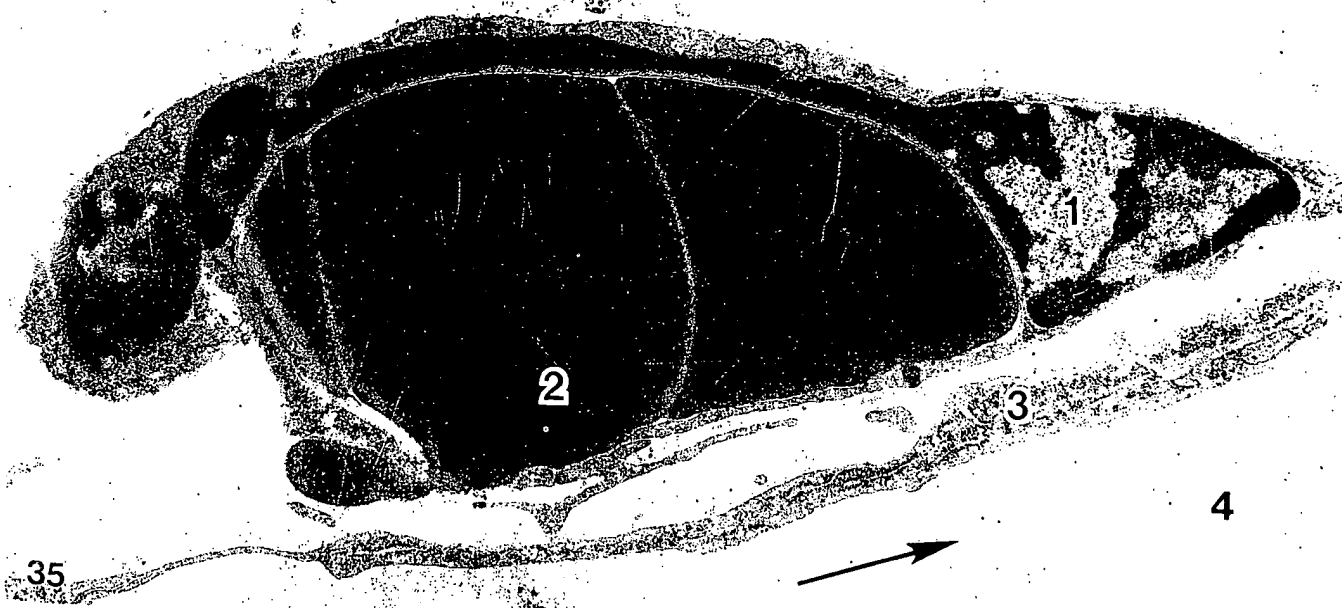
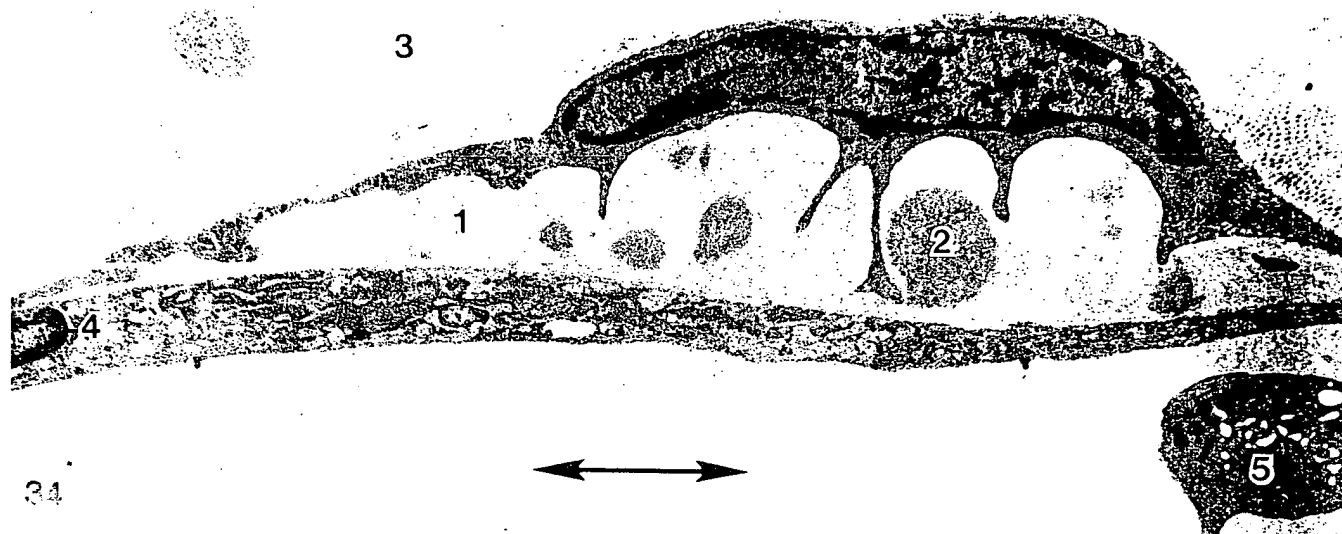
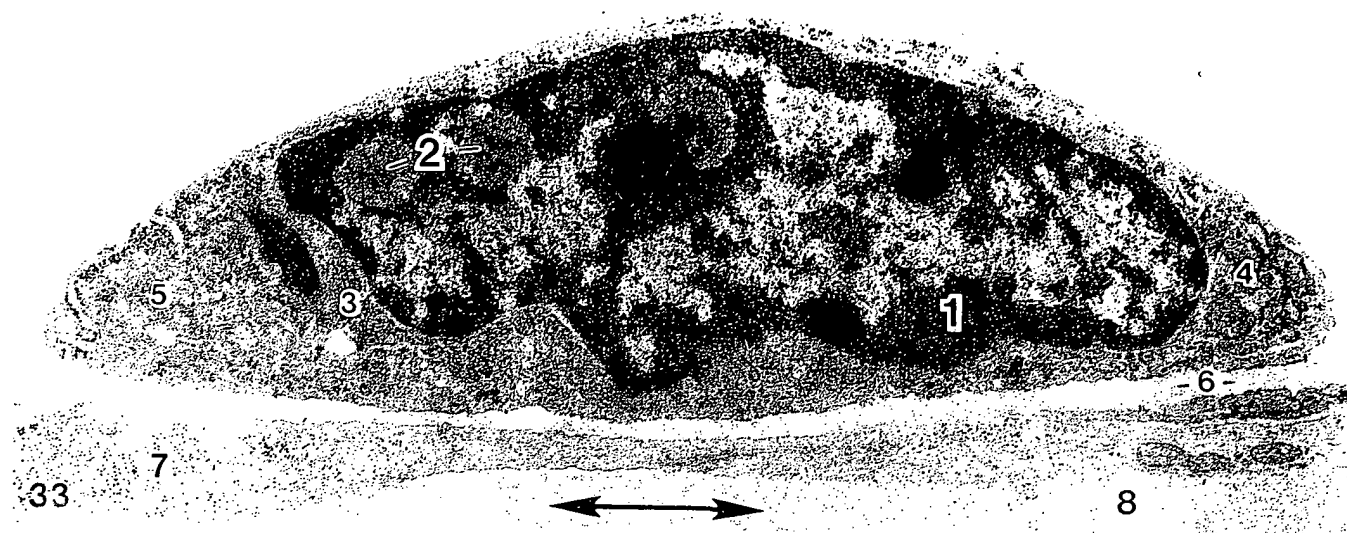
obtained (Fig. 35) which mimicked so-called 'seamless' capillaries with erythrocytes in the 'lumen' (Wolff, 1964; Wolff and Bär, 1972). However, serial sections proved them to be pericytes with trapped erythrocytes. The subpericytic space also contained wispy or round profiles of fibrin deposits. The fate of the trapped items is still not clear, since there was no indication in our material that the pericytes did phagocytose the material. It is very likely that the formed elements of the blood disintegrated extracellularly through a lytic process, since 'ghosts' of erythrocytes were frequently encountered in the subpericytic space.

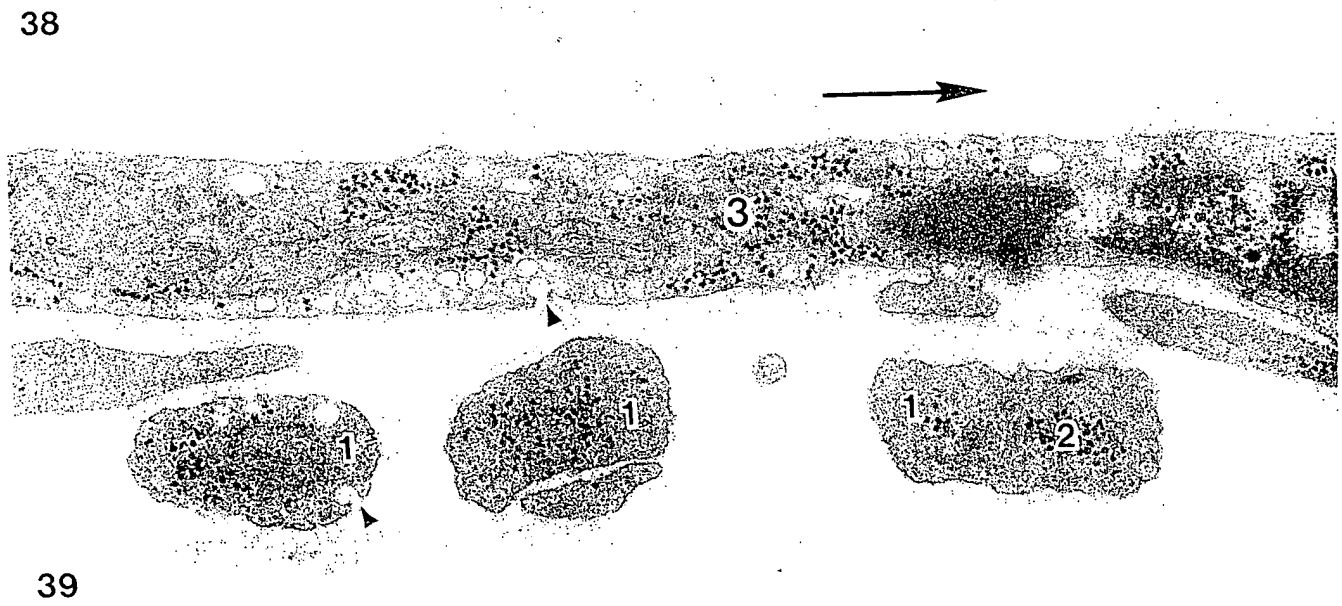
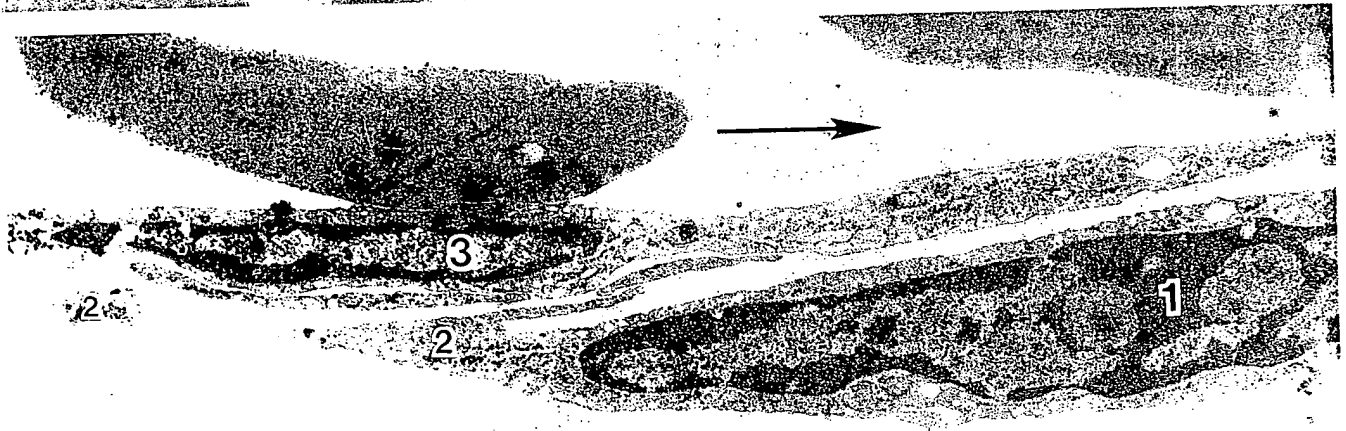
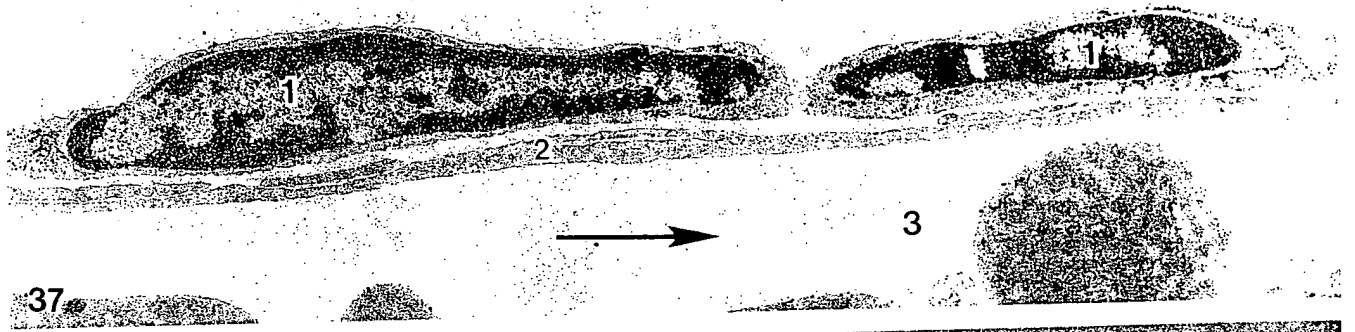
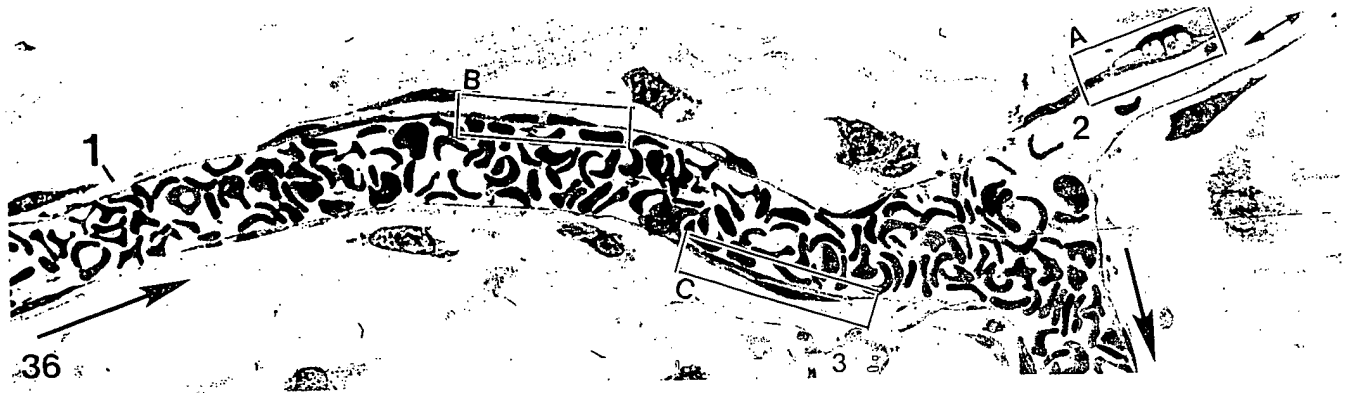
It has also been suggested that pericytes are *precursors of smooth muscle cells* (Meyrick *et al.*, 1981). In our material, it was observed that the perivascular cells of the arteriolar feeder of the arteriolar-venular loop (Fig. 36) were similar to the so-called intermediate cells which we described earlier (Rhodin, 1968). These cells had characteristics which made them look more like smooth muscle cells (Fig. 37) than pericytes, since they were situated more around the vessel with long, slender (Fig. 38) regularly spaced, cytoplasmic branches (Fig. 39) circling the vessel at right angle to its long axis. Cytoplasmic processes of pericytes were irregularly spaced and ran in many directions, many times parallel to the long axis. The cytoplasm of pericytic processes contained a mixture of intermediate filaments, microtubules and ribosomes, whereas the intermediate cell processes had a dense cytoplasm with many

FIGURE 33 Enlargement of a pericyte associated with the capillary sprout area seen in rectangle in Fig. 13. Typically, pericytes have a pronounced heterochromatic nucleus (1) with several nucleoli (2), which makes them easy to detect already intravitaly. The cytoplasm has a smaller supply of ribosomes (3) and rough endoplasmic reticulum (4) than a fibroblast, and the overall higher cytoplasmic density is caused mostly by a larger number of intermediate filaments, but the Golgi area (5) is smaller, and there are fewer plasmalemmal vesicles. The pericyte has a basal lamina which is sometimes fused with the endothelial basal lamina (6). The sprout endothelium (7) is less dense than that of the pericyte. There is no blood flow in the lumen (8) of capillary sprouts but oscillating pulsations (bidirectional arrow) of the plasma column in synchronization with the heart beats. $\times 18,400$. $1 \text{ mm} = 0.054 \mu\text{m}$.

FIGURE 34 A pericyte associated with the proximal part of a long capillary sprout. It has assumed an umbrella-shape during the process of holding back flocculent plasma (1) which leaked out through detached endothelial cell junctions (not shown) of the fragile capillary sprout. Fibrinoid material (2) is also held back and prevented from escaping into the perivascular interstitium (3). An endothelial nucleus (4) is seen, as well as a platelet in the lumen (5) of the sprout which showed oscillating pulsations of the plasma column intravitaly (bidirectional arrow). The precise location of this pericyte is seen in the upper right corner of Fig. 36, rectangle marked 'A'. $\times 9,800$. $1 \text{ mm} = 0.01 \mu\text{m}$.

FIGURE 35 A pericyte with a highly compressed nucleus (1) associated with the arterial capillary marked 'A' in Fig. 48. This particular capillary showed a pronounced extravasation of erythrocytes, and associated pericytes had engulfed one or several erythrocytes (2) in a process reminiscent of the action of phagocytes. However, actual phagocytosis did not take place, since the pericytes lacked lysosomes. The erythrocytes disappear through some kind of lysis, since red cell ghosts are frequently found. This pericyte mimics a 'seamless' capillary at some level of the more than 100 serial sections of this area, but the same technical procedures did confirm its true nature of being a pericyte. Endothelial cytoplasm (3) and vascular lumen (4). Arrow indicates direction of blood flow. $\times 13,600$. $1 \text{ mm} = 0.074 \mu\text{m}$.





intermediate filaments and only few microtubules and ribosomes.

Vascular anastomosis

Earlier investigations contributed very little to answering the questions of how capillary sprouts find other sprouts or capillaries, and what cell components participate in the merger. This investigation did not solve the problem but certain findings seemed relevant to the issue. It must be emphasized that the present observations were based on light and electron microscope analyses of static images. Therefore, it was difficult to ascertain whether a vascular sprout was moving toward or retracting from a certain microvascular segment.

Two areas of assumed potential merger were investigated in serial sections. One dealt with a sprout with a blunt leading tip situated within $1\text{ }\mu\text{m}$ from the venular leg of an arteriolar-venular loop (Fig. 41). It was assumed that the blunt end was in the process of achieving contact with the loop rather than retracting from it, since there were no signs of cellular breakdown, such as nuclear pyknosis or lysis of organelles in the endothelial cells of the sprout tip. The entire sprout displayed features very similar to those described above under 'long blunt sprouts'.

Platelets and erythrocytes were not present in the lumen, which was somewhat wider at the distal, leading end with several small bleb-like out-pocketings. The nuclei of the endothelial cells of the blunt leading end were large and irregularly shaped (Fig. 45). The cytoplasm displayed many slender, as well as bulbous extensions (Fig. 46), some of which were within $0.1\text{ }\mu\text{m}$ distance from the endothelial cells of the adjacent arteriolar-venular loop, although none had established a membranous contact. A basal lamina was absent in the area of the blunt end. All extensions of the endothelial cells contained numerous microsomes and profiles of rough endoplasmic reticulum. The endothelial cells had large vesicular Golgi areas, prominent centrioles, and small nests of highly electron-dense whirls of smooth membranes, as well as numerous microtubules and intermediate filaments. The endothelial cells of the adjacent arteriolar-venular loop did not display cytoplasmic projections toward the blunt end of the sprout (Fig. 41). Based on these observations, it was concluded that this particular sprout was still in an active but slow phase of growth, and it is very likely that the endothelial cell extensions would soon have made membranous contacts with the endothelial cells of the arteriolar-venular loop.

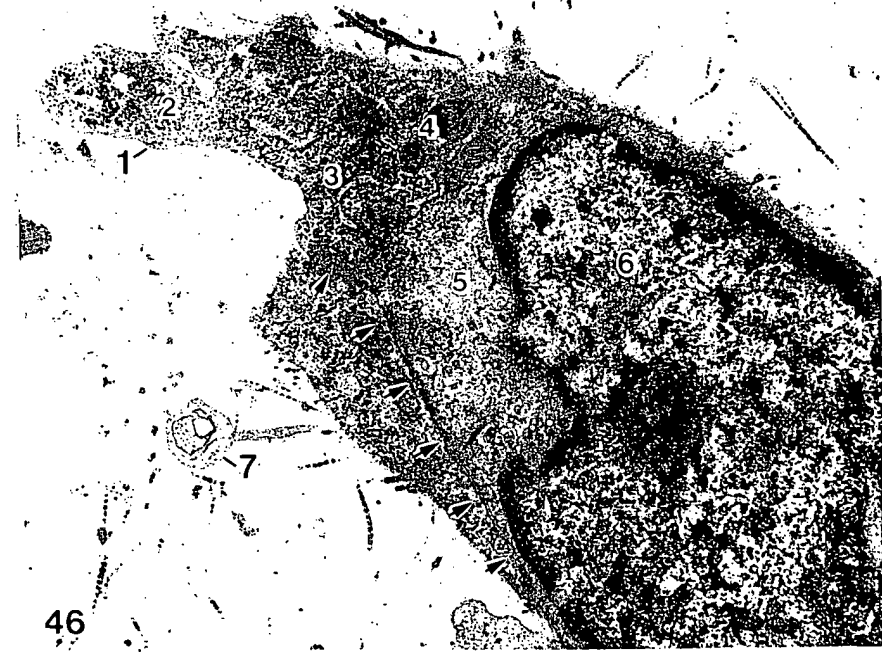
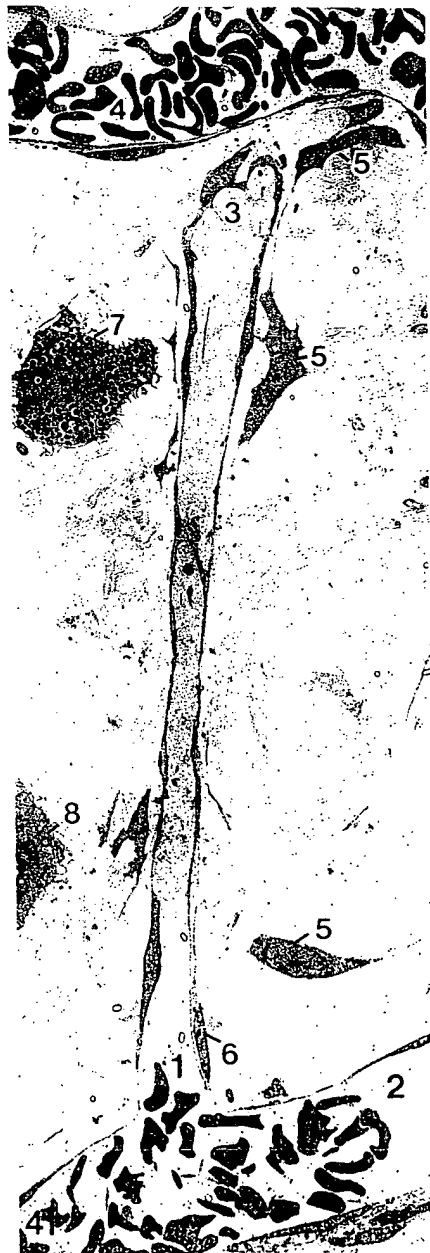
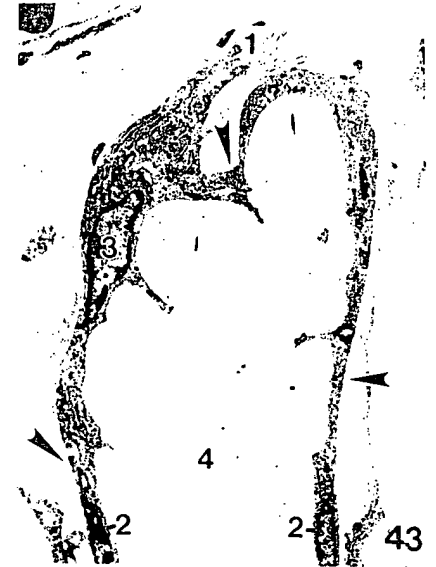
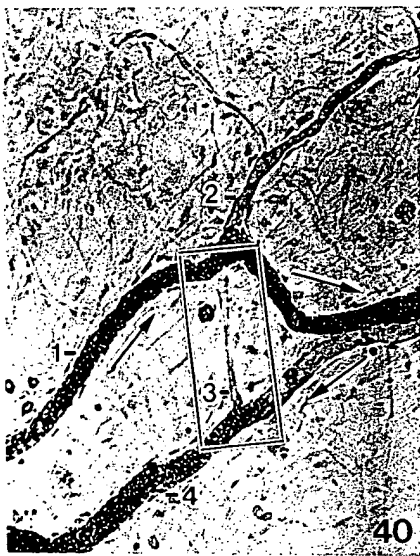
The second area of assumed merger dealt with two very

FIGURE 36 Electron microscopic overview of the arterial feeder of the arteriolar-venular loop, marked '2' in Fig. 11. The overall width of the arterial feeder (1) is $16\text{ }\mu\text{m}$. It is assumed that this arteriolar-venular loop started out as a capillary loop and that its component segments are in the process of being transformed into an arteriole and a venule of increasingly larger diameters. The proximal end of a long capillary sprout (2) and the proximal end of another sprout (3) are seen. Rectangle 'A' is enlarged in Fig. 34, rectangle 'B' in Fig. 37, and rectangle 'C' in Figs. 38 and 39. Single arrow indicates direction of blood flow. Bidirectional arrow indicates oscillating movements of plasma column recorded in the sprout. $\times 865$. $1\text{ mm} = 1.2\text{ }\mu\text{m}$.

FIGURE 37 Enlargement corresponding to area within rectangle 'B' in a different serial section demonstrates two intermediate cells (1), the thin endothelial lining (2) and the lumen (3) of this vascular segment. The intermediate cells show characteristics typical for pericytes, as well as smooth muscle cells. They are flatter than the typical pericyte seen in Fig. 33. They have a smaller amount of rough endoplasmic reticulum, and their orientation is more perpendicular to the long axis of the vessel. Arrow indicates flow direction. $\times 16,000$. $1\text{ mm} = 0.063\text{ }\mu\text{m}$.

FIGURE 38 Enlargement of area within rectangle 'C' in Fig. 36. The nucleus (1) of this intermediate cell is more bulgy and the orientation of the cell more parallel to the long axis of the vessel, making its appearance closer to that of a pericyte. Extensions of the intermediate cell (2) approach and surround the endothelial cell (3) in the typical fashion of a smooth muscle cell. Arrow indicates blood flow. $\times 19,500$. $1\text{ mm} = 0.051\text{ }\mu\text{m} = 51\text{ }\mu\text{m}$.

FIGURE 39 Enlargement of the extended left region in Fig. 38. The structural appearance of the cytoplasm of the intermediate cell extensions (1) is dense because of numerous microfilaments and a paucity of cell organelles, except ribosomes (2). Plasmalemmal vesicles (arrowheads) are associated with these extensions as well as with the endothelial cell (3). It is assumed that the extensions of the intermediate cell are gradually transformed into cytoplasmic processes typical for a vascular smooth muscle cell. $\times 46,000$. $1\text{ mm} = 0.022\text{ }\mu\text{m} = 22\text{ nm} = 220\text{ }\text{\AA}$.



short sprouts which originated from a long sprout and were arching back and did achieve a merger with their own microvascular segment of origin (Figs. 47 to 52). In the video playbacks, it was observed that there was a slow and oscillating stream of plasma and platelets moving into the long sprout 'A' which grew out from the arteriolar-venular loop. At the very end of the fixation period, three erythrocytes moved into sprout 'A'. It was also observed that plasma was flowing into sprout 'B', since occasional platelets found their way from sprout 'A' into sprout 'B'. With reference to sprout 'C', it was impossible to ascertain the direction of plasma flow, if any, since no platelets were observed to move in or out of the sprout connection with the arteriolar-venular loop. The analysis of serial sections in the electron microscope (Figs. 53 to 55) revealed a discontinuous part of sprout 'C' whereby the lumen of the arteriolar-venular loop communicated with a connective tissue channel lined by a delicate basal lamina and pericytes, filled with plasma

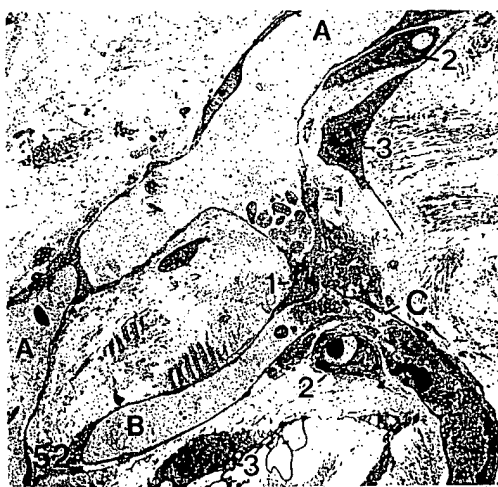
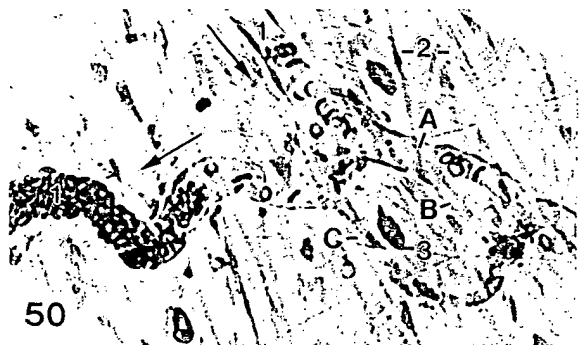
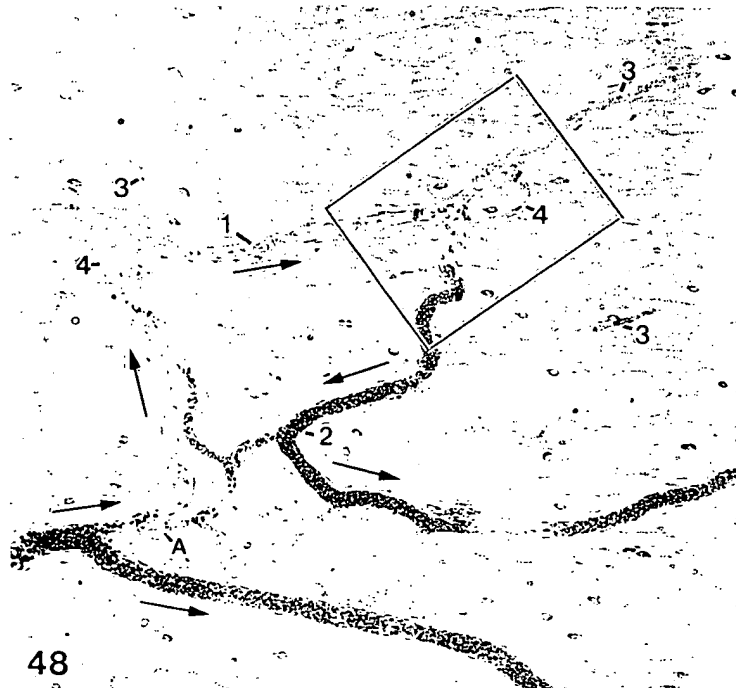
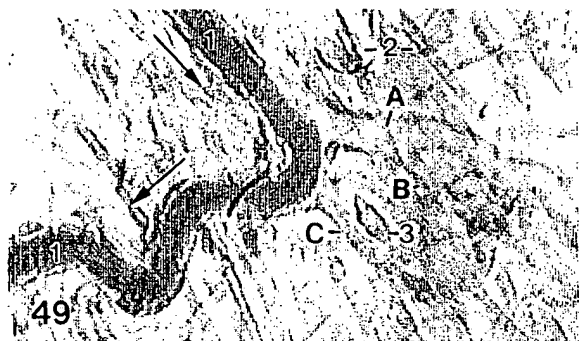
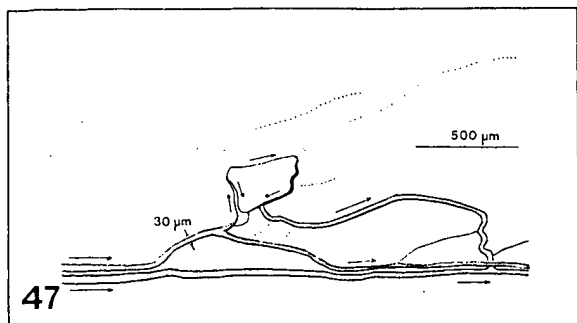
and some platelets, as well as several firmly lodged erythrocytes (Figs. 53 and 55). The red blood cells were also seen very clearly in the video playbacks of the loops (Fig. 50). It was difficult to decide based on static images in serial sections, which part of sprout 'C' was the leading end, and which was the point of origin. Based on the flow pattern in these microvessels there was a strong flow in the arteriolar-venular loop, and very likely, this stimulated the outgrowth of an endothelial spur from the loop. The blood pressure may then have caused the discontinuity of the short sprout, leaking plasma and platelets into the interstitial 'channel'. Sprouts 'A', 'B' and 'C' shared endothelial cells (Fig. 52), and it is very likely that sprout 'C' therefore was an outgrowth from sprout 'A' moving toward the merger with the discontinuous endothelial spur from the arteriolar-venular loop. Based on these observations, it was concluded that an actual process of sprout merger may initially involve disruption of the fragile leading tip, with extravasation of plasma and

FIGURE 40 Same microvascular area seen in Fig. 11. From the arterial feeder (1) of the arteriolar-venular loop arises a branched capillary sprout (2). A shorter sprout (3) takes its origin from the venous drainer (4). Arrows indicate direction of blood flow. The leading tip of this sprout is analyzed in selected sections in Figs. 42 to 46 from a sequence of 163 thin serial sections. $\times 192$. 1 mm = 5.2 μm .

FIGURE 41 Electron microscopic overview of the rectangle in Fig. 40. The sprout is 114 μm long and 4.8 μm wide. Its proximal part (1) arises from the venous drainer (2) and it is assumed that the distal leading end (3) is seeking contact with the arterial feeder (4) in order to establish a vascular anastomosis. Perivascular cells are fibroblasts (5), a pericyte (6), a mast cell (7) and a macrophage (8). This is section No. 93 from a sequence of 163 serial sections of these vascular segments. $\times 1,100$. 1 mm = 0.91 μm .

FIGURES 42 to 45 Selective serial sections of leading sprout tip in Fig. 41, illustrating cell modulations as the sprout moves forward and prepares for an anastomosis with an adjacent vascular segment. The distal endothelial surface is highly irregular projecting differently shaped endothelial pseudopodia (1) in a forward motion. The nuclei are elongated (2), large and highly irregular (3) interpreted as a sign of polyploidy and/or nuclear movement. Several endothelial cells make up the leading sprout tip. Careful analysis of many serial sections at low and high magnification revealed distinct cell borders and junctional areas (arrowheads), and the fact that some of the nuclei (3) seen at certain levels (Figs. 43 and 44) only represent one nucleus (Fig. 45). The lumen (4) is dilated all the way to the tip, which is typical for long blunt sprouts. At certain levels of sectioning, a bleb appears (5) to be situated within an endothelial cell, but at other levels, it is clearly seen to represent an outpocketing of the sprout lumen (4). The lumen develops as a dilatation of the interendothelial space, and in this process, adhering remnants (6) of cytoplasmic strands are the last to become detached. These are sections No. 115 (Fig. 42), 88 (Fig. 41), 79 (Fig. 44), and 65 (Fig. 45) from a sequence of 163 serial sections of this area. $\times 3,700$. 1 mm = 0.27 μm .

FIGURE 46 Enlargement of area corresponding to rectangle in Fig. 45 in yet another serial section (No. 59). The pseudopodium (1) contains numerous ribosomes (2), rough endoplasmic reticulum (3), mitochondria (4), and there is a highly vesicular Golgi area (5) near the nucleus (6). A microspike with a ball-shaped tip (7) is devoid of basal lamina in similarity with the pseudopodium (1). Junctional areas of apposing cell membranes (arrowheads) indicate the place where the sprout lumen appears as a bleb marked '5' in Fig. 45. $\times 13,700$. 1 mm = 0.073 μm .



formed elements until firm and less primitive cell junctions have been established between endothelial cells.

DISCUSSION

This investigation dealt with the extension and further development of blood vessels in a mammalian system after the primary differentiation has taken place and after the circulation has been established. Furthermore, it dealt with a situation where the vascular growth did occur under quite normal circumstances in young rats without previous experimental intervention or damage to the tissue such as, for instance, cauterization, inflammation, scar tissue formation or observation of growth in ear chambers. The microvessels of the mesentery were under continuous growth and development in young rats, since the mesenteric membrane was used for fat deposition, and we assumed that this deposition required a microvascular system for its sustenance and exchange of lipid molecules.

Technical approach

The investigation utilized integrated intravital video recording and light and transmission electron microscopy of

the same vascular segments in serial sections. Thus, there was an absolutely positive identification of not only the vascular segments that were observed by video recording, but also of component cells and their topographic relationship. Intravital observations of microvascular beds are commonplace (Chambers and Zweifach, 1944; Zweifach, 1954, 1973; Buckley and Ryan, 1969; Fox and Wayland, 1979; Fox *et al.*, 1980; Weigelt and Schwartzmann, 1981; Zawicki *et al.*, 1981; Hashimoto and Prewitt, 1987), and it is relatively easy to ascertain flow direction as well as the precise location and identification of a given vascular segment within the microvascular bed. The preservation of the microvessels for light and electron microscopy occurs while observing and video-recording the microvascular bed. Events that take place during fixation were observed, recorded and thoroughly evaluated (Rhodin, 1986). Microvascular segments were analyzed before, during and moments after superfusion fixation with glutaraldehyde. Invariably, the flow in the arteriolar-venular loops slowed down within 20-40 sec and came to a complete stop before the end of 60 sec. The diameter of the loops as well as the base of the capillary sprouts remained unchanged, but it was often noticed that erythrocytes and platelets would enter the lumen of the proximal end of the sprout to a larger extent than was the case during the pre-fixa-

FIGURE 47 Tracing of a microvascular area in a 4 week-old rat mesentery. The flow direction in arterioles and arterial capillaries (red) as well as in venules and venous capillaries (blue) was confirmed by intravital video recording. Capillary sprouts (dotted yellow-green) showed oscillating plasma columns.

FIGURE 48 Microvessels present within green rectangle in Fig. 47 seen in a light micrograph of a fixed and Epon embedded whole-mount before sectioning. This demonstrates a rather restricted area of a microvascular bed with one arteriolar (1)-venular (2) loop, several capillary sprouts (3) and two capillary sprout loops (4) in stages of formation and establishment of vascular anastomoses. Arrows indicate blood flow direction. $\times 125$. 1 mm = 1.1 μ m.

FIGURE 49 Intravital video image of rectangular area in Fig. 48, recorded 10 min before the blood flow was stopped by superfusion fixation with glutaraldehyde. There was a rapid, continuous blood flow in the arteriolar-venular loop (1), but only plasma and an occasional platelet entered sprout A and sprout B (poorly resolved) suspended in oscillating plasma columns. No platelets were seen to enter sprout C from the arteriolar-venular loop. Note collagen fibers (2) and the mast cell (3). Arrows indicate blood flow direction. $\times 350$. 1 mm = 2.9 μ m.

FIGURE 50 Same area as Fig. 49 seen in a light micrograph of a whole-mount after fixation and Epon embedding but before sectioning. Three erythrocytes entered sprout A during the last 20 sec before the blood flow was arrested by the fixative. The general topography corresponds well to that recorded intravital as evidenced by the close correlation of the arteriolar-venular loop (1), the collagen fibers (2) as well as the mast cell (2). The outlines of the short loops and their sprout components (A, B, C) are better defined than in Fig. 49. $\times 350$. 1 mm = 2.9 μ m.

FIGURE 51 Electron microscopic overview of the area indicated by a rectangle in Fig. 48. The arterial feeder (1) as well as the venous drainer of the arteriolar-venular loop (2) have an average 15 μ m in width. Their walls are made up of thin endothelial cells (3) and scattered intermediate-type cells (4) (resolved as such at higher magnifications). Sprout A which is wide at its origin, contains several platelets and two erythrocytes. Sprout B does not appear in its entirety in this section, but does connect with sprout C as seen in Fig. 52. Sprout C is at the initial stages of formation and is analyzed further in Figs. 53 to 55. Pericytes (5), fibroblasts (6) and mast cells (7) and an 'umbrella cell' (8) were positively identified at higher magnification. $\times 900$. 1 mm = 1.1 μ m.

FIGURE 52 Serial section to Fig. 51 demonstrates the connection between the sprouts A, B and C seen at this level of sectioning. The endothelial cell with its nucleus (1) shares cytoplasm with all three sprouts, thus establishing an anastomosis between them. Many platelets and some erythrocytes are blocking the connective tissue channel or incomplete lumen of sprout C (compare Figs. 51 and 52) thus preventing blood from flowing through. Two pericytes (2) have assumed the shape of 'umbrella cells' (see Figs. 34 and 35), and the greater extent of two fibroblasts (3) is seen. $\times 1,350$. 1 mm = 0.74 μ m.

tion period (Fig. 50). Based on earlier investigations (Rhodin, 1986) and those made during this investigation, one is assured of the fact that the general topography of the microvascular bed as well as the vascular dimensions are preserved in the state that existed just prior to the onset of the fixation.

It is quite difficult, if not impossible, to ascertain with complete confidence that the capillary sprouts observed were in the process of growth and development. The mesentery of the rat can be observed for only a very limited time, and actual growth could not be seen during the 60-120 min of recording. Therefore, other criteria were used to decide that we were, indeed, dealing with active capillary growth, rather than retraction or regression. The extensive and elegant studies by the Clarks (1912, 1918, 1925, 1934, 1939, 1940, 1943), which dealt with *in vivo* observations of capillary growth in frog larvae tail fins and the rabbit ear chamber, served as elucidating guides in identifying vascular sprouts in our material, since the Clark observations were made during prolonged periods of up to several days, even weeks. It would be very appropriate to repeat some of the Clark investigations, using modern observation methods (Allen *et al.*, 1981; Shotton, 1987; Aletta and Greene, 1988), including time-lapse photography in order to determine whether a capillary sprout was involved in a migratory movement toward another capillary, or was in a process of regression and atrophy. In our present investigation for all intents and purposes, we had to use other criteria, such as the absence of intracellular lysis of cell organelles and nuclear pyknosis to rule out capillary retraction. Furthermore, investigations by Schoefl (1963), Ausprunk and Folkman (1977), Folkman and Haudenschild (1980), Wagner (1980),

Montesano *et al.* (1963), Sholley *et al.* (1984), and Dvorak *et al.* (1987) dealing with vascular sprouting and proliferation of endothelial cells during a variety of experimental conditions and using different investigative tools, were very helpful in identifying morphological signs of angiogenesis in the mesentery of our material. These signs included shape and size of endothelial cell extensions breaking through the basal lamina; bipolar rearrangement of migrating endothelial cells; emergence of a primitive sprout lumen; shape and topographic relationship of leading sprout tip to adjacent mast cells, macrophages and fibroblasts; and the ultrastructural composition and arrangement of cell organelles in the migrating bipolar endothelial cells.

Mechanisms of sprouting

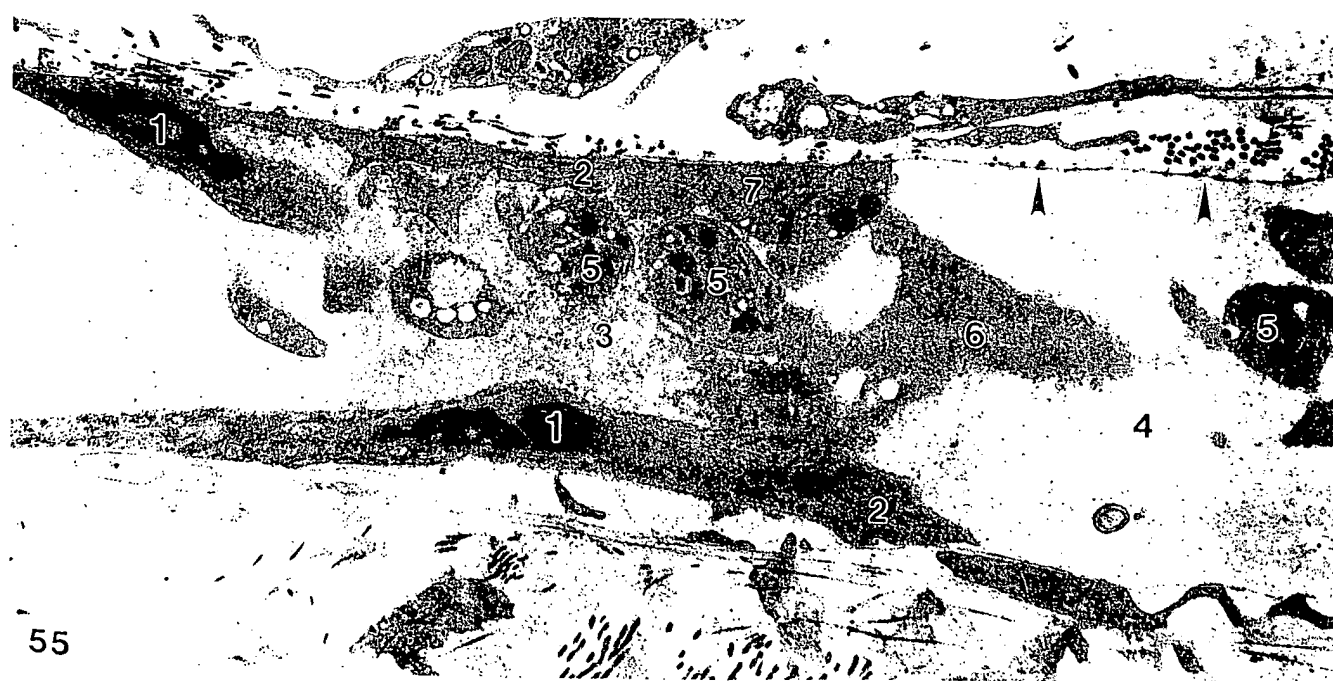
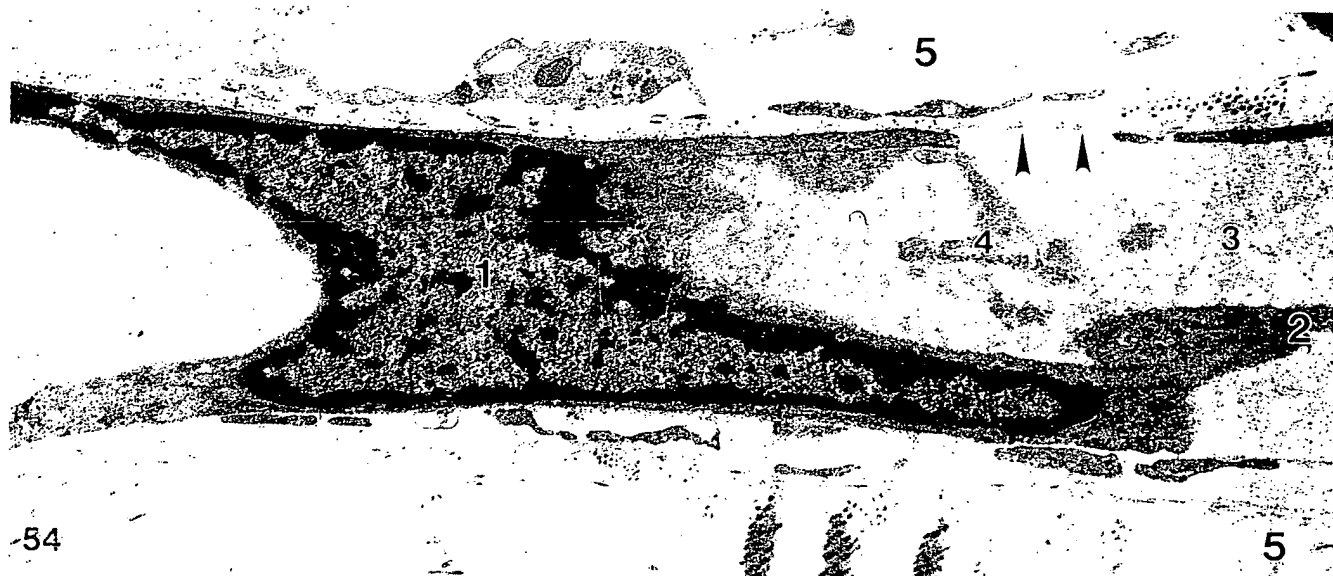
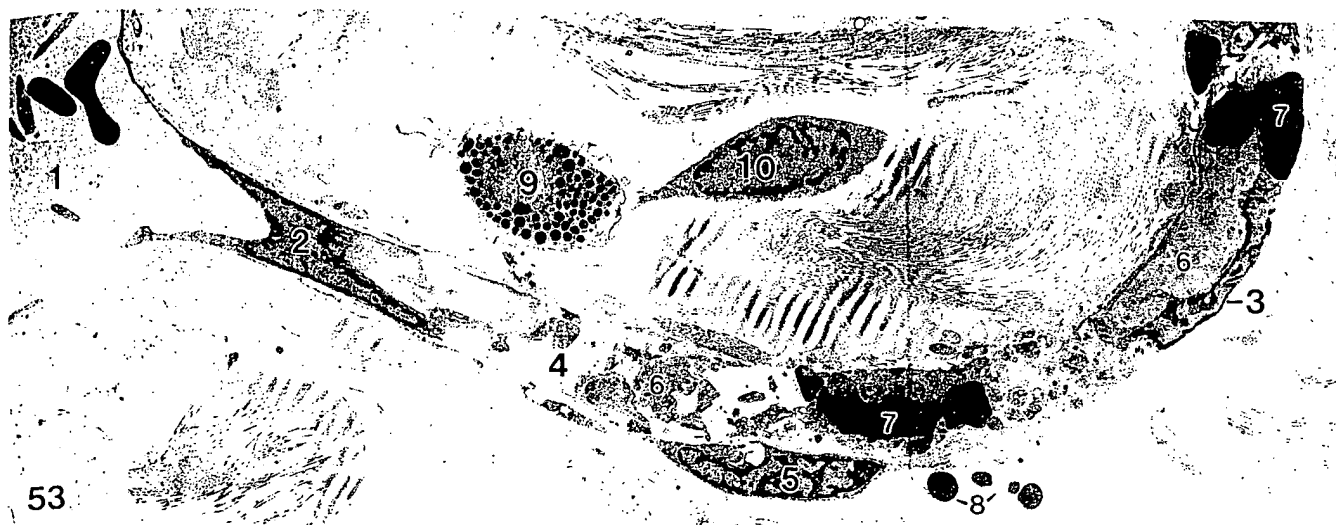
The mechanisms of capillary growth consist of several factors and phases such as blood flow and growth factor related stimuli, endothelial cell proliferation (mitosis), endothelial cell migration, intraendothelial migration devices, endothelial and interstitial connective tissue factors.

Blood flow related stimuli - In our present material from the rat mesentery, the majority of capillary sprouts studied did arise from the general area of arteriolar-venular loops, mostly at the sharp angle where the loop changed from an arteriolar feeder into a venous drainer (Figs. 1 to 4). The video recordings indicated clearly that platelets and erythrocytes, suspended in the plasma column at the basal (proximal) end of the sprout, showed oscillatory movements in synchrony with the heart beats. Gradually, the middle and distal part of the sprouts became filled with packed erythrocytes and platelets as the plasma es-

FIGURE 53 Enlarged detail of sprout C in Fig. 51. From the bend of the arteriolar-venular loop (1) emerges an endothelial spur together with its nucleus (2). Another endothelial cell with its nucleus (3) is assumed to approach the spur from the opposite direction. An interstitial channel (4), partly lined by a pericyte (5), contains platelets (6) and erythrocytes (7), the latter escaping into the perivascular connective tissue with their leading ends (8). The perivascular cells are a mast cell (9) and a fibroblast (10). $\times 2,250$. 1 mm = 0.44 μ m.

FIGURE 54 Enlargement of the endothelial spur with its nucleus (1) in Fig. 53. Serial sections showed that there is only one endothelial cell making up the spur. The distal end of the spur is frayed (2) but the endothelial basal lamina (arrowheads) remains intact, separating the flocculent plasma (3) and fibrinoid (4) from the interstitial connective tissue (5). $\times 9,500$. 1 mm = 0.11 μ m.

FIGURE 55 In another serial section of this area, it is convincingly demonstrated that the frayed appearance of the endothelial spur seen in Fig. 54 is due to an open, disrupted endothelial cell (1) spur tip (2) allowing plasma (3) to create and fill a connective tissue channel (4). Platelets (5) also escape into this channel, and fibrinoid (6) is formed. One platelet (7) lines up protectively along the denuded basal lamina (arrowheads). $\times 12,000$. 1 mm = 0.083 μ m.



caped to the interstitial space. It is suggestive that these oscillations and the packing of erythrocytes would have a stimulating effect on the vascular growth, as proposed by Brånemark (1965). Furthermore, we observed a rapid blood flow in the arteriolar-venular loops. Clark *et al.* (1930), Myrhage and Hudlicka (1978) and Hudlicka (1984) have postulated that high blood flow and/or blood pressure may have a stimulating effect on capillary growth, possibly through shear stress in the angular regions of capillaries. It is presently not known how these factors would influence capillary growth and whether they would have a mitogenic effect on the endothelial cells, activate intraendothelial cytoplasmic structures involved in cell elongation and migration, or have a multifactorial effect.

Growth factors - It is beyond the scope of this communication to discuss the influence of growth factors on capillary sprouting, since our investigations did not include biochemical assays. As demonstrated by Maciag *et al.* (1984) the principal endothelial cell mitogen in bovine brain is a growth factor which is bound by heparin. For a comprehensive review, consult Hormia and Virtanen (1986).

Endothelial cell migration - One important phase of capillary growth is the migration of endothelial cells. Based on our observations, we postulate that the migration process is divided into a rapid phase and a slow phase. The rapid phase is represented by the sprouts with long, narrow, pointed leading tips without lumen, and the slow phase by the sprouts with blunt thick ends and an open, dilated lumen. During the *rapid phase*, the cytoplasm and the nucleus of the endothelial cells became elongated and the cells polarized. The leading pointed part was provided with small filopodia and bulbous pseudopodia which broke through the basal lamina, while the trailing part established contact and differentiated temporary junctional areas with accompanying endothelial cell(s). Based on our serial sections of cross-sectioned leading ends of long sprouts, we arrived at the conclusion that the mechanism by which the migration of endothelial cells occurs during the rapid phase of capillary growth is through a sliding movement. Thus, one endothelial cell moves forward rapidly, followed by a second, elongated bipolar endothelial cell, in a sliding, overlapping motion. Whether one or the other endothelial cell overtakes its companion during the sliding could not be determined on the basis of our investigation. However, our concept is in line with an earlier suggestion (Bär, 1983) that the elongation process of developing microvessels in the cerebral cortex of rats takes place through a telescope-like sliding of the margins of overlapping endothelial cells. A similar mechanism was suggested by Sholley *et al.* (1984) in their study of vascular

lar sprouting in the rat cornea following silver nitrate cauterization, as well as by Hadfield (1951), who investigated granulation tissue. Gradually, more than two endothelial cells participated in the build-up of the middle and proximal parts of the capillary sprouts. As a small and slit-like lumen emerged, it did so between endothelial cells. There was no indication, in our material, that the sprout lumen developed through intraendothelial vacuolization, as was suggested by Clark and Clark (1930), giving rise to so-called seamless endothelia, described by Wolff (1964) and Güldner and Wolff (1973) in a variety of tissues and organs.

During the *slow phase* of capillary growth, we assume that the endothelial cells which make up the leading, blunt end of the sprout, are undergoing some kind of reorganization. This is based on the fact that the nuclei of those endothelial cells are large and irregularly shaped, in contrast to the elongated and smooth nuclei of the polarized endothelial cells of the sprouts with the long leading tip. Although mitotic figures were not recorded in these cells, it is likely that the nuclei are in an early prophase, or engaged in DNA synthesis, leading to polyploidy. Should this be the case, an ensuing cell division or nuclear enlargement could result in a rapid phase of capillary growth by endothelial cell migration. The fairly large number of closely apposed fibroblasts near the leading blunt end of these sprouts and their multiple contacts through cytoplasmic peg-and-socket devices may have an arresting influence on endothelial cell proliferation, as suggested by Gospodarowicz *et al.* (1980) and Orlidge and D'Amore (1987), since some of the fibroblasts are probably in the stage of transforming into pericytes. On the other hand, the many bulbous pseudopodia of the endothelial cells of the blunt sprout end indicate that the cells do prepare to move on (Aletta and Green, 1988). The suggested subdivision of the endothelial migration process into rapid and slow phases is purely speculative, and must be verified by time-lapse photography, preferably utilizing video-enhanced high-resolution differential interference contrast microscopy (Allen *et al.*, 1981; Shotton, 1987), in a vascular sprouting system, where one can observe the development of the microvascular bed over prolonged periods. The model used by Clark (1918), the tail fin of amphibian tadpoles, seems to offer unprecedented opportunities for such a time-lapse recording, and should yield valuable information in support of or against our theory.

Endothelial cell proliferation - Mitotic figures were not observed with great frequency in the endothelial cells of the capillary sprouts studied in our material. Since nearly all fibroblasts often underwent cell division, we assume that our fixation technique is adequate to preserve cells in mi-

osis. In a few instances, it was observed that the endothelial cells near the proximal end of long sprouts underwent division. It is well known that endothelial cells of fully developed blood vessels, including capillary endothelial cells, have a very slow turn-over rate (Engerman *et al.*, 1967). On the other hand, more recent investigations by Shollev *et al.* (1984) demonstrated clearly by means of tritiated thymidine and autoradiography that the endothelial cell nuclei of capillary sprouts in the cauterized rat cornea were engaged in DNA synthesis and subsequent mitosis.

In our material, it was noticed that the nuclei of the endothelial cells located within the most distal, leading part of the vascular sprouts were unusually elongated, reaching lengths of up to 25 μm , and that they were not observed in mitosis. There is the possibility that the elongated endothelial cells are in a state of DNA replication without karyokinesis and cytokinesis. Thus, the cells become polyploid during their most active migratory phase. This may be an adaptive device, since the completion of cell division during this stage might be disruptive to the intracellular organelles which aid in the migratory movement of the endothelial cells. Support for this theory is found in the investigation by Black *et al.* (1988) into the question of smooth muscle cell polyploidy in the blood vessels of spontaneously hypertensive rats. However, a more definitive answer to the question of polyploidy versus mitosis in the endothelial cells of sprouting capillaries will require a combination of our technical approach and labeling by tritiated thymidine in the mesentery of young rats.

Intercellular migration devices - As the sprout grows forward, it is inevitable that the endothelial cells which make up the sprout actively bring about this movement, perhaps in a sliding motion in relation to their companion cells, as suggested above. The endothelial cells of the sprouts become polarized during the assumed rapid migratory phase, and the distal end is highly drawn out and provided with small microspikes and bulbous cytoplasmic extensions, piercing through the basal lamina of the sprout and into the interstitial matrix. The polarized endothelial cells are richly endowed with organelles that, in other mammalian cells and other species, are known to participate in migratory movements. The leading part of the polarized endothelial cells contains many microtubules, small vesicles and filaments. Our filaments average 75 Å in diameter and are, therefore, on the borderline between microfilaments (60 Å-70 Å) and intermediate filaments (80 Å-100 Å). To determine their protein composition and thus, their functions, other investigative methods are required, for instance immunofluorescence (Blose, 1981). True microfilaments, also referred to as

'stress fibers' (White and Fujiwara, 1986; Herman and D'Amore, 1984) are made up of F-actin (Lazarides and Weber, 1974; Wong *et al.*, 1983) and myosin (Fujiwara and Pollard, 1976; Weber and Gröschel-Stewart, 1974), whereas intermediate filaments are chemically heterogeneous. In cells of mesenchymal origin such as fibroblasts, pericytes and endothelial cells, the backbone of intermediate filaments is vimentin, a 50,000-dalton protein (Gard and Lazarides, 1980). Microfilaments are generally considered to participate in cytoplasmic contractions, cellular adhesion and anti-shear stress. Vimentin filaments form an important supportive and strengthening part of the cytoskeleton. The 75 Å filaments observed in our material are either intermediate filaments or microfilaments, or they may represent yet a different type of filament which may be transitory in nature, serving some special function only during cell migration. The presence also of numerous microtubules (Dustin, 1984) and vesicles in the long endothelial extensions makes it tempting to formulate the following scenario, supported by several recent investigations of nerves and nerve growth cones (Bunge, 1973, 1986; Schnapp *et al.*, 1985; Allen, 1987; Aletta and Green, 1988), locomotion of cultured fibroblasts (Bretscher, 1984, 1987), pollen tubes of angiosperms (Picton and Steer, 1982; Pierson *et al.*, 1986) and reticulopods of protozoans (Allen, 1985, 1987; Allen *et al.*, 1985; Bowser *et al.*, 1988; Travis *et al.*, 1983).

During the migration of the endothelial cells of the capillary sprouts, the Golgi area, unusually rich in vesicular components, generates a large number of small cytoplasmic vesicles, and the centriolar region serves as origin for the many microtubules (Gudima *et al.*, 1988) present in the distal leading edge of the polarized endothelial cell. Thus, the leading extension contains a system of microtubules and 75 Å filaments arranged in parallel and cytoplasmic vesicles strung along together with mitochondria, microsomes and some multivesicular bodies and secondary lysosomes. In the locomotion, the cytoplasmic vesicles and other organelles are transported along by the microtubules, aided by the 75 Å filaments which also provide structural integrity to the cytoplasmic matrix. At the leading tip, the cytoplasmic vesicles add membranous material to the cell membrane through exocytosis, thus ultimately making the tip move forward. The microspikes and lamellipodia of this area are probably created by an increase in cell membrane area, although their penetration of the basal lamina of their parent endothelial cell and their probing ahead through the extracellular connective tissue matrix is very likely brought on through the influence of factors released from mast cells and other sources.

In summary, it is quite remarkable to observe the close similarity in shape and structural composition between

the growth cone of nerves and the distal leading endothelial process of capillary sprouts. However, other investigations are needed to ascertain the biochemical nature of the 75 Å filaments observed in our material, as well as to confirm that the postulated cytoplasmic streaming and transport does, indeed, take place in endothelial sprouts during their migration and growth.

Extraendothelial factors - There are several extraendothelial factors which participate in capillary growth. The basal lamina of the sprout is synthesized by the endothelial cells and serves primarily as a filtration barrier and supporting layer between the endothelial cell and the surrounding connective tissue. However, it is dissolved at the moment the endothelial cell projects pseudopodic extensions into the interstitial connective tissue (Glaser *et al.*, 1983). The polypeptide chains which make up laminin, the major component of the basal lamina, are probably dissolved through the action of proteases, enzymes derived from the mast cells (Folkman *et al.*, 1983). Other enzymes, also synthesized by the mast cells (Klebe *et al.*, 1986), are very likely responsible for the degradation of mucopolysaccharides in the ground substance (Fraser and Simpson, 1983), thus creating the interstitial connective tissue channels which we observed in our material. One such channel can be seen in Figs. 51 and 52, and it should be noted that there is also an intact 'nude' basal lamina present, marking the border between the connective tissue channel and the surrounding connective tissue. In this instance, the basal lamina serves not only as a barrier but perhaps also as a scaffold for the continued growth and migration of the endothelial cell.

It is difficult to decide which factors have an influence on the direction of the sprout growth. Of course, growth factors act on cell membrane receptors (Liu and Newell, 1988), and interaction between specific cell membrane domains and connective tissue components will promote and help in moving the endothelial extensions forward (Thiery, 1984). The close proximity between endothelial extensions and elastic fibers demonstrated by us may indicate that these fibers serve as a scaffold for the migration, thus, indirectly, serving as directional guides for capillary growth as suggested by Mortimer *et al.* (1983) and Ryan and Barnhill (1983).

Relationships between fibroblasts, pericytes and smooth muscle cells - This investigation has shed some new light on the possible developmental connection between the fibroblasts of the mesenteric membrane, the pericytes of the capillary sprouts, and the emergence of primitive smooth muscle cells on the arteriolar-venular arcades during vascular growth and differentiation in the rat mesentery. The work by Clark and Clark (1925) which analyzed the development of adventitial cells on the blood capil-

laries of amphibian larvae, demonstrated quite convincingly that fibroblasts in the tail fin of these larvae moved about by ameboid movements. Some of the fibroblasts settled down on newly formed capillaries, often going through a mitotic division first, and then pulled in many of their long cytoplasmic processes and gradually became cells which, by all morphological evidence, must be called pericytes. However, many of the fibroblasts that moved about did not settle down and become pericytes. More recently, Cliff (1963, 1965) studied the kinetics of wound healing in rabbit ear chambers by time-lapse cinematography and electron microscopy, and showed that adventitial, fibroblast-like cells of developing arterioles were derived from extravascular cells, which gradually developed into smooth muscle cells. Similarly, Meyrick *et al.* (1981) demonstrated in the small pulmonary arteries in the rat lung that pericytes do differentiate into smooth muscle cells.

Our investigation showed that perivascular fibroblasts invariably 'close in' on capillary endothelial spurs and short sprouts, and continue to do so as the sprouts grow and lengthen. There is a bilateral activity between these fibroblasts and the endothelial cells as evidenced by the many peg-and-socket contacts established. Perhaps the most convincing evidence of a transformation of a fibroblast into a pericyte is seen in Figs. 29, 30 and 32, where we, presumably, captured a fibroblast in the process of surrounding the leading tip of a long capillary sprout. Thus, we maintain based on the findings in this investigation, that we have demonstrated beyond a doubt that fibroblasts do serve as precursors of pericytes. Furthermore, we have good evidence that pericytes serve as precursors of smooth muscle cells, as seen in Figs. 37 to 39, showing cells located in the wall of the arteriolar feeder of an arteriolar-venular loop.

Thus, the fibroblasts are transformed into pericytes, and these into smooth muscle cells during capillary growth and differentiation of the newly formed capillary loops into arterioles and venules.

The function of the bilateral peg-and-socket connections between the fibroblasts and the endothelial cells is probably the first indication of a cell-mediated impulse, whereby the two cells exchange information related to their continued collaborative efforts in capillary growth (Heimark and Schwartz, 1985). The sprout must secure pericytes which will serve as progenitors of future smooth muscle cells. However, the acquisition of pericytes may also serve to coordinate endothelial activities, as suggested by Crocker *et al.* (1970) based on their electron microscope analysis of the role of pericytes in wound healing. Recently, Orlidge and D'Amore (1987) showed that pericytes suppress or modulate endothelial growth *in vitro*. Thus, the incorporation of fibroblasts and their transfor-

DEVELOPMENT OF CAPILLARY SPROUTS (rat mesentery)

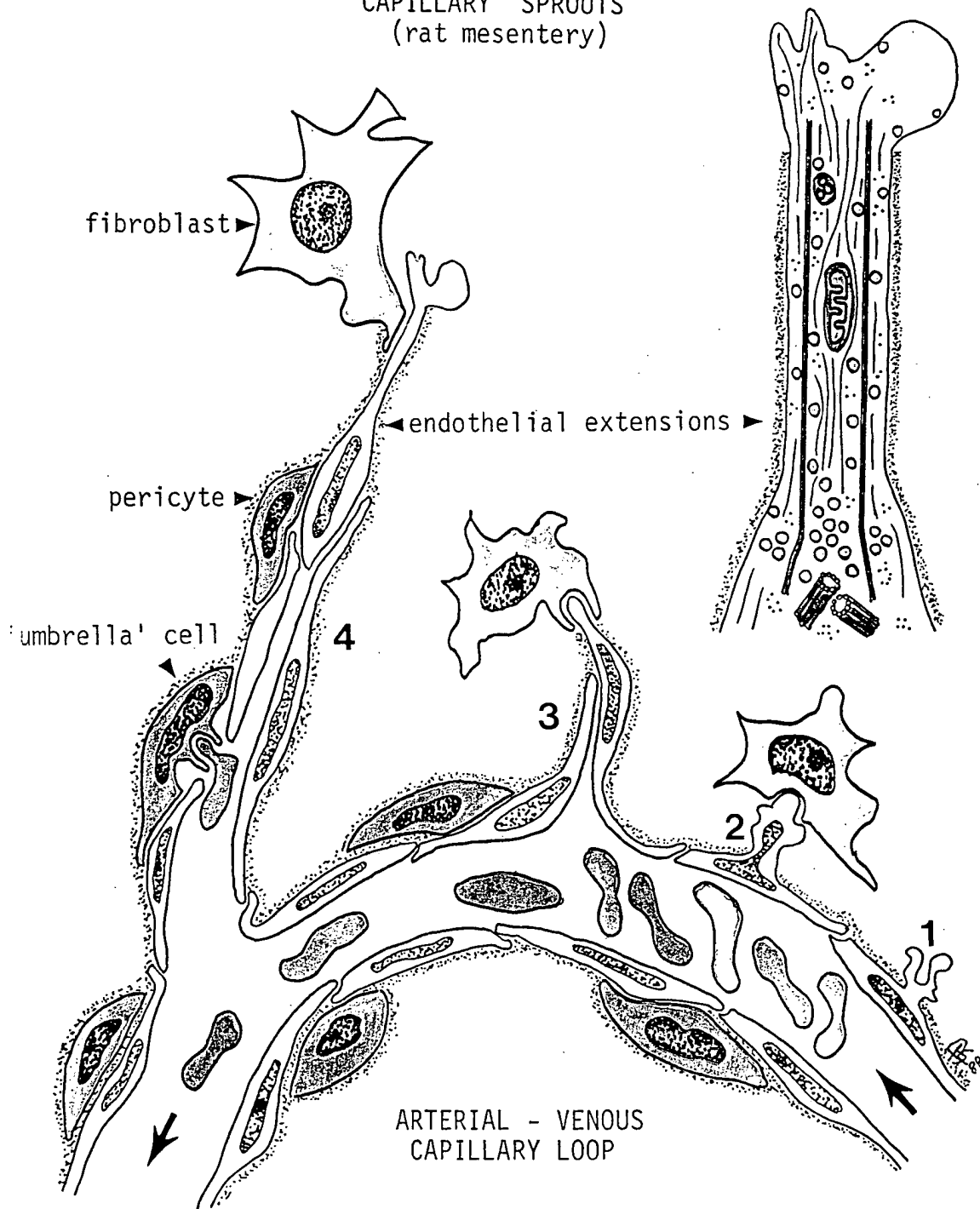


FIGURE 56 Schematic drawing summarizing the major events taking place during the growth and development of capillary sprouts, based on analyses of intravital video recordings and electron micrographs from the mesenteric microcirculation of young rats. In arterial-venous capillary loops, small endothelial extensions (1) penetrate the basal lamina (fine stippling), evolve into cellular protrusions (2) and develop further into endothelial spurs and short sprouts (3). Gradually, the sprouts lengthen (4). The long endothelial extensions (yellow) contain a highly vesicular Golgi area which delivers a multitude of vesicles (orange) which are transported distally by cytoplasmic streaming, aided by 7.5 nm filaments (black wavy lines) and a set of microtubules, derived from the centrosomes (red). Other cell components are mitochondria and lysosomes/multivesicular bodies (green) as well as monoribosomes (black dots). The vesicles fuse with the cell membrane, thereby adding membranous material to the endothelial extensions and making the sprout tip move forward. Fibroblasts (lavender) settle down on the sprouts and are transformed into pericytes (green). Extravasation of plasma (pale rose) and formed elements of the blood (red) are prevented from escaping into the interstitial tissue by pericytes which temporarily assume an umbrella shape.

mation into pericytes may delay or arrest temporarily endothelial cell proliferation, as evidenced by the virtual absence of mitotic figures in the endothelial cells of the distal, leading end of capillary sprouts.

Yet another function of the pericytes is evidenced by the fact that they serve as reinforcements of the very delicate and quite leaky wall of the capillary sprouts. They accumulate plasma and extravasated formed elements of the blood, temporarily transforming the primitive pericytes into umbrella-shaped cells. This function of serving as modulators of vascular leakage is retained in the fully developed microvascular bed, where this function is particularly well demonstrated in the postcapillary venules (Majno and Palade, 1961; Majno *et al.*, 1961; Cotran, 1965; Hultström and Svensjö, 1979; Sims *et al.*, 1985; Sims, 1986) this being the most sensitive segment, responding to histamine release as well as thermal and inflammatory stimuli.

Vascular anastomosis

The very limited number of observed and assumed potential mergers between capillary sprouts and their arteriolar-venular loops of origin or with other capillary loops do not warrant a lengthy discussion. Time-lapse video recordings of such vascular fields followed by detailed electron microscope analyses are necessary prerequisites, and would enable one to decide whether a given capillary sprout is approaching or retracting from another capillary. Our present observations seem to indicate that interstitial channels are created during the process of vascular anastomosis before firm contacts have been established between merging endothelial cells, allowing for a flow of plasma and formed elements of the blood through connective tissue channels without endothelial lining, rather than through endothelial tubes.

CONCLUSIONS

The formation of capillary sprouts and their further growth and development was explored in the mesentery of young rats without experimental intervention. The mechanisms of sprout formation and migration are very similar to capillary growth in wound healing. The mesentery, therefore, offers a unique opportunity to study angiogenesis as close to its natural state as possible.

The principle consecutive stages of the mesenteric capillary growth are summarized in the schematic drawing in Fig. 56. They are as follows:

- 1) Endothelial projections penetrate the basal lamina.
- 2) Endothelial nucleus moves into the projection.
- 3) An endothelial 'spur' is formed.
- 4) Matrix 'channels' form in the interstitial connective tissue in advance of the approaching capillary sprout.
- 5) Endothelial cells migrate by an alternate sliding motion, in the process assuming an extreme bipolarity.
- 6) The leading sprout tip consists of microspikes and pseudopodia, devoid of a basal lamina.
- 7) Intraendothelial components participating in the endothelial migration are the Golgi area, which delivers numerous small vesicles which are assumed to move peripherally into the distal endothelial extension, and a system of microtubules and 7.5 nm filaments, arranged in parallel, which offers a mechanism by which vesicles, mitochondria and multivesicular bodies are moved along in a cytoplasmic streaming within the leading endothelial extension, thus ultimately moving the endothelial cell forward.
- 8) The sprout follows a connective tissue scaffold of elastic fibers and collagen fibers.
- 9) A sprout lumen is gradually formed between endothelial cells.
- 10) Fibroblasts move toward the sprouts and 'cradle' the extensions of the endothelial cells.
- 11) Gradually, the fibroblasts are transformed into pericytes, and in the process are being surrounded by a basal lamina.
- 12) The pericytes first serve to protect the delicate and fragile capillary sprouts, preventing a major exodus of plasma and formed elements of the blood into the connective tissue. In the process, the pericytes take on an umbrella shape.
- 13) Gradually, the intercellular junctions between the endothelial cells of the capillary sprouts become firmer, and fewer vascular elements 'leak' out. The 'umbrella cell' resumes the shape of ordinary pericytes.
- 14) As the arterial-venous capillary loops grow in size, and develop into arterioles and venules, the pericytes are transformed into intermediate cells, gradually acquiring cytoplasmic characteristics typical of vascular smooth muscle cells.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the American Heart Association and with funds contributed in part by the AHA Florida Affiliate. Preliminary intravital analyses were performed in the Department of Anatomy, Osaka University, Osaka, Japan, and in part supported by Japan Society for the Promotion of Science through a Fellowship to J.A.G. Rhodin in 1983. The authors wish to thank Ms. Margaret Bryant for printing the illustrations and Mrs. Betsey Stiepoek for typing the manuscript.

REFERENCES

- ALETTA J.M. and GREENE L.A., 1988. Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. *J. Neurosci.*, 8, 1425-1435.
- ALLEN R.D., 1985. New observations on cell architecture and dynamics by video-enhanced contrast optical microscopy. *Annu. Rev. Biophys. Biophysic. Chem.*, 14, 265-290.
- ALLEN R.D., 1987. The microtubule as an intracellular engine. *Sci. Am.*, 257, 149-157.
- ALLEN R.D., ALLEN N.B. and TRAVIS J.L., 1981. Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: a new method capable of analysing microtubule-related motility in the reticulopodial network of *Allogromia laticollaris*. *Cell Motil.*, 1, 291-302.
- ALLEN R.D., WEISS D.G., HAYDEN J.H., BROWN D.T., FUJIWAKE H. and SIMPSON M., 1985. Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport. *J. Cell Biol.*, 100, 1736-1752.
- ALLEN R.D., D.H. and FOLKMAN J., 1977. Migration and proliferation of endothelial cells in preformed and newly formed vessels during tumor angiogenesis. *Microvasc. Res.*, 14, 53-65.
- BÄR T., 1983. Patterns of vascularization in the developing cerebral cortex. In: 'Development of the vascular system'. *Ciba Found. Symp.*, 100, 20-36.
- BLACK M.J., CAMPBELL J.H. and CAMPBELL G.R., 1988. Does smooth muscle cell polyploidy occur in resistance vessels of spontaneously hypertensive rats? *Blood Vessels*, 25, 89-100.
- BLOSE S.H., 1981. The distribution of 10 nm filaments and microtubules in endothelial cells during mitosis: double-label immunofluorescence study. *Cell Motil.*, 1, 417-431.
- BOWSER S.S., TRAVIS J.L. and RIEDLER C.L., 1988. Microtubules associate with actin-containing filaments at discrete sites along the ventral surface of *Allogromia reticulopods*. *J. Cell Sci.*, 89, 297-307.
- BRÄNEMARK P.I., 1965. Capillary form and function. *Bibl. Anat.*, 7, 9-28.
- BRETSCHER M.S., 1984. Endocytosis: relation to capping and cell locomotion. *Science*, 224, 681-686.
- BRETSCHER M.S., 1987. How animal cells move. *Sci. Am.*, 257, 72-90.
- BRETSCHER M.S., I.K. and RYAN G.B., 1969. Increased vascular permeability. *J. Pathol.*, 55, 329-347.
- BUNGE M.B., 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.*, 56, 713-735.
- BUNGE M.B., 1986. The axonal cytoskeleton: its role in generating and maintaining cell form. *Trends Neurosci.*, 9, 477-482.
- CHAMBERS R. and ZWEIFACH B.W., 1944. Topography and function of the mesenteric circulation. *Am. J. Anat.*, 75, 173-205.
- CLARK E.R., 1912. Further observations on living growing lymphatics: their relation to the mesenchymal cells. *Am. J. Anat.*, 13, 351-379.
- CLARK E.R., 1918. Studies on the growth of blood-vessels in the tail of frog larvae by observation and experiment on the living animal. *Am. J. Anat.*, 23, 37-88.
- CLARK E.R. and CLARK E.L., 1925. The development of adventitial (Rouget) cells on the blood capillaries of amphibian larvae. *Am. J. Anat.*, 35, 239-264.
- CLARK E.R. and CLARK E.L., 1934. Observations on living arterio-venous anastomoses as seen in transparent chambers introduced into the rabbit's ear. *Am. J. Anat.*, 54, 229-286.
- CLARK E.R. and CLARK E.L., 1939. Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.*, 64, 291-299.
- CLARK E.R. and CLARK E.L., 1940. Microscopic observations in the extra-endothelial cells of living mammal. *Am. J. Anat.*, 66, 1-49.
- CLARK E.R. and CLARK E.L., 1943. Caliber changes in minute blood-vessels observed in the living mammal. *Am. J. Anat.*, 73, 215-250.
- CLARK E.R., KIRBY-SMITH H.T., REX R.O. and WILLIAMS R.G., 1930. Recent modifications in the method of studying living cells and tissues in transparent chambers inserted in the rabbit's ear. *Anat. Rec.*, 47, 187-211.
- CLIFF W.J., 1963. Observations on healing tissue: a combined light and electron microscopic investigation. *Philos. Trans. R. Soc. London (Biol. Sci.)*, 246, 305-325.
- CLIFF W.J., 1965. Kinetics of wound healing in rabbit ear chambers, a time lapse cinemicroscopic study. *Q. J. Exp. Cogn. Med. Sci.*, 50, 79-89.
- COTRAN R.S., 1965. The delayed and prolonged vascular leakage in inflammation. II. An electron microscopic study of the vascular response after thermal injury. *Am. J. Pathol.*, 46, 589-620.
- CROCKER D.J., MURAD T.M. and GEER J.C., 1970. Role of pericyte in wound healing. An ultrastructural study. *Exp. Mol. Biol.*, 13, 51-65.
- DUSTIN P., 1984. 'Microtubules. Cell Shape. Cell Movement'. Springer Verlag, Berlin, pp. 234-293.
- DVORAK H.F., HARVEY V.S., ESTRELLA P.E., BROWN L.F., McDONAGH J. and DVORAK A.A., 1987. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. *Lab. Invest.*, 57, 673-686.
- ENGERMAN R.L., PFAFFENBACH D. and DAVIS M.D., 1967. Cell turnover of capillaries. *Lab. Invest.*, 17, 738-743.
- FOLKMAN J. and HAUDENSCHILD C., 1980. Angiogenesis *in vitro*. *Nature*, 288, 551-556.
- FOLKMAN J., TAYLOR S. and SPILLBERG C., 1983. The role of heparin in angiogenesis. In: 'Development of the Vascular System'. *Ciba Found. Symp.*, 100, 132-149.
- FOX J. and WAYLAND H., 1979. Interstitial diffusion of macromolecules in the rat mesentery. *Microvasc. Res.*, 18, 255-276.
- FOX J., GALEY F. and WAYLAND H., 1980. Action of histamine on the mesenteric microvasculature. *Microvasc. Res.*, 19, 108-126.
- FRASER R.A. and SIMPSON J.G., 1983. Role of mast cells in experimental tumor angiogenesis. In: 'Development of the vascular system'. *Ciba Found. Symp.*, 100, 120-149.
- FUJIWARA K. and POLLARD T.D., 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.*, 71, 848-875.
- GARD D. and LAZARIDES F., 1980. The synthesis and distribution of desmin and vimentin during myogenesis *in vivo*. *Cell*, 19, 263-275.
- GLASER B.M., KALEBIC T., GARBISA S., CONNOR T.B. and LIOTTA L.A., 1983. Degradation of basement membrane components by vascular endothelial cells: role in neovascularization. In: 'Development of the vascular system'. *Ciba Found. Symp.*, 100, 150-162.
- GOSPODAROWICZ D., VLODAVSKY I. and SAVION N., 1980. The extracellular matrix and the control of proliferation of vascular endothelial and vascular smooth-muscle cells. *J. Supramol. Struct.*, 13, 339-372.
- GUDIMA G.O., VOROBJEV I.A. and CHENTSOV Y.S., 1988. Centriolar location during blood cell spreading and motion *in vitro*: an ultrastructural analysis. *J. Cell Sci.*, 89, 225-241.
- GÜLDNER F.H. and WOLFF J.R., 1973. Seamless endothelia as indicators of capillaries developed from sprouts. *Bibl. Anat.*, 12, 120-123.
- HADFIELD G., 1951. Granulation tissue. *Ann. R. Coll. Surg. Engl.*, 9, 397-407.
- HASHIMOTO H. and PREWITT R.L., 1987. Microvascular density changes during wound healing. *Int. J. Microcirc. Clin. Exp.*, 5, 303-310.
- HEIMARK R.L. and SCHWARTZ S.M., 1985. The role of membrane-membrane interactions in the regulation of endothelial cell growth. *J. Cell Biol.*, 100, 1934-1940.
- HERMAN I.M. and D'AMORE P.A., 1984. Capillary endothelial cell migration: loss of stress fibres in response to retina-derived growth factor. *J. Muscle Res. Cell Motil.*, 5, 697-709.

- HORMIA M. and VIRTANEN I., 1986. Endothelium - an organized monolayer of highly specialized cells. *Med. Biol.*, **64**, 247-266.
- HUDLICKA O., 1984. Development of microcirculation: capillary growth and adaptation. In: 'Handbook of Physiology, Sect. 2. The Cardiovascular System'. American Physiological Society, Bethesda, Maryland, vol. IV, pp. 165-216.
- HULTSTRÖM D. and SVENSJÖ E., 1979. Intravital and electron microscopic study of bradykinin-induced vascular permeability changes using FITC-dextran as a tracer. *J. Pathol.*, **129**, 125-133.
- HÜTTNER I., JELLINEK H. and KERENYI T., 1968. Fibrin formation in vascular fibrinoid change in experimental hypertension: an electron microscopic study. *Exp. Mol. Pathol.*, **9**, 309-231.
- KLEBE R.J., ESCOBEDO L.V., BENTLEY K.L. and THOMPSON L.K., 1986. Regulation of cell motility, morphology, and growth by sulfated glycosaminoglycans. *Cell Motil. Cytoskeleton*, **6**, 273-281.
- LAZARIDES E. and WEBER K., 1974. Actin antibody: the specific visualization of actin filaments in non-muscle cells. *Proc. Natl. Acad. Sci. USA*, **71**, 2268-2272.
- LIU G. and NEWELL P.C., 1988. Evidence that cyclic GMP regulates myosin interaction with the cytoskeleton during chemotaxis of *Dictyostelium*. *J. Cell Sci.*, **90**, 123-129.
- MACIAG T., MEHLMAN T., FRIESEL R. and SCHREIBER A.B., 1984. Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science*, **225**, 932-935.
- MAJNO G. and PALADE G.E., 1961. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *J. Biophys. Biochem. Cytol.*, **11**, 571-605.
- MAJNO G., PALADE G.E. and SCHOEFL G.I., 1961. Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographic study. *J. Biophys. Biochem. Cytol.*, **11**, 607-626.
- MEYRICK B., FUJIWARA K. and REID L., 1981. Smooth muscle myosin in precursor and mature smooth muscle cells in normal pulmonary arteries and the effect of hypoxia. *Exp. Lung Res.*, **2**, 303-313.
- MONTESANO R., ORCI L. and VASSALLI P., 1983. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.*, **97**, 1648-1652.
- MORTIMER P.S., CHERRY G.W., JONES R.L., BARNHILL R.L. and RYAN T.L., 1983. The importance of elastic fibers to skin lymphatics. *Br. J. Dermatol.*, **108**, 561-566.
- MOVAT H.Z. and MORE R.H., 1957. The nature and origin of fibrinoid. *Ann. J. Clin. Pathol.*, **28**, 331-353.
- MYRHAGE R. and HUDLICKA O., 1978. Capillary growth in chronically stimulated adult skeletal muscle as studied by intravital microscopy and histological methods in rabbits and rats. *Microvasc. Res.*, **16**, 73-90.
- ORLIDGE A. and D'AMORE P.A., 1987. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.*, **105**, 1455-1462.
- PICTON J.M. and STEER M.W., 1982. A model for the mechanism of tip extension in pollen tubes. *J. Theor. Biol.*, **98**, 15-20.
- PIERSON E.S., DERKSEN J. and TRAAS J.A., 1986. Organization of microfilaments and microtubules in pollen tubes grown in vitro or in vivo in various angiosperms. *Eur. J. Cell Biol.*, **41**, 14-18.
- RHODIN J.A.G., 1967. The ultrastructure of mammalian arterioles and precapillary sphincters. *J. Ultrastruct. Res.*, **18**, 181-223.
- RHODIN J.A.G., 1968. The ultrastructure of mammalian venous capillaries, venules and small collecting veins. *J. Ultrastruct. Res.*, **25**, 452-500.
- RHODIN J.A.G., 1972. Fixation of microvasculature for electron microscopy. *Microvasc. Res.*, **5**, 285-291.
- RHODIN J.A.G., 1974. 'Histology: a Text and Atlas'. Oxford University Press, New York.
- RHODIN J.A.G., 1980. Architecture of the vessel wall. In: 'Handbook of Physiology, Sect. 2, The Cardiovascular System'. American Physiological Society, Bethesda, Maryland, vol. II, pp. 1-31.
- RHODIN J.A.G., 1984. Anatomy of microcirculation. In: 'Blood Vessels and Lymphatics in Organ Systems'. Academic Press, New York, pp. 97-106.
- RHODIN J.A.G., 1986. Perfusion and superfusion fixation effects on rat mesentery microvasculature beds. Intravital and electron microscope analyses. *J. Submicrosc. Cytol.*, **18**, 453-470.
- RYAN T.J. and BARNHILL R.L., 1983. Physical factors and angiogenesis. In: 'Development of the Vascular System'. *Ciba Found. Symp.*, **100**, 80-94.
- SCHNAPP B.J., VALE R.D., SHEETZ M.P. and REESE T.S., 1985. Single microtubules from squid axoplasm support bidirectional movements of organelles. *Cell*, **40**, 455-462.
- SCHOEFL G.I., 1963. Studies on inflammation. III. Growing capillaries: their structure and permeability. *Virchows Arch. Pathol. Anat.*, **337**, 97-141.
- SHOLLEY M.M., FERGUSON G.P., SEIBEL H.R., MONTGOMERY J.L. and WILSON F.D., 1984. Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells. *Lab. Invest.*, **51**, 624-634.
- SHOTTON D., 1987. The current renaissance in light microscopy. I. Dynamic studies of living cells by video enhanced contrast microscopy. *Proc. R. Microsc. Soc.*, **22**, 37-44.
- SIMS D.E., 1986. The pericyte - a review. *Tissue Cell*, **18**, 153-174.
- SIMS D.E., MILLER F. and PERRICONE M.A., 1985. A role of pericyte in skeletal muscle vasculature during macromolecular leakage. *Microvasc. Res.*, **29**, 250 (Abstr).
- THIERY J.P., 1984. Review - Mechanisms of cell migration in the vertebrate embryo. *Cell Differ.*, **15**, 1-15.
- TILTON R.G., KILO C. and WILLIAMSON J.R., 1979. Pericyte-endothelial relationships in cardiac and skeletal muscle capillaries. *Microvasc. Res.*, **18**, 325-335.
- TRAVIS J.L., KNEALY J.F.X. and ALLEN R.D., 1983. Studies on the motility of the foraminifers. II. The dynamic microtubular cytoskeleton of the reticulopodial network of *Allogromia laticollaris*. *J. Cell. Biol.*, **97**, 1668-1676.
- WAGNER R.C., 1980. Endothelial cell embryology and growth. *Adv. Microcirc.*, **9**, 45-75.
- WEBER K. and GRÖSSEL-STEWART U., 1974. Antibody to myosin: specific visualization of myosin-containing filaments in nonmuscle cell. *Proc. Natl. Acad. Sci. USA*, **71**, 4561-4564.
- WEIGELT H. and SCHWARZMANN V., 1981. A new method for the simultaneous presentation of low and high magnification of microscopic specimens: application to in vivo studies of mesenteric capillaries. *Microsc. Acta*, **85**, 161-173.
- WHITE G.E. and FUJIWARA K., 1986. Expression and intracellular distribution of stress fibers in aortic endothelium. *J. Cell Biol.*, **103**, 63-71.
- WOLFF J., 1964. Ein Beitrag zur Ultrastruktur der Blutkapillaren: cnahtlose Endothel. *Z. Zellforsch.*, **64**, 290-300.
- WOLFF J.R. and BÄR T., 1972. 'Seamless' endothelia in brain capillaries during development of the rat's cerebral cortex. *Brain Res.*, **41**, 17-24.
- WONG A.J., POLLARD T.D. and HERMAN I.M., 1983. Actin filament stress fibers in vascular endothelial cells in vivo. *Science*, **219**, 867-869.
- ZAWICKI D.F., JAIN R.K., SCHMID-SCHOENBEIN G.W. and CHIEN S., 1981. Dynamics of neovascularization in normal tissue. *Microvasc. Res.*, **21**, 27-47.
- ZWEIFACH B.W., 1954. Direct observations of mesenteric circulation in experimental animals. *Anat. Rec.*, **120**, 277-288.
- ZWEIFACH B.W., 1973. The microcirculation in the interstitial mesentery. *Microvasc. Res.*, **5**, 363-367.

The Mouse Gene for Vascular Endothelial Growth Factor

GENOMIC STRUCTURE, DEFINITION OF THE TRANSCRIPTIONAL UNIT, AND CHARACTERIZATION OF TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATORY SEQUENCES*

(Received for publication, October 20, 1995)

David T. Shima†§, Masatoshi Kuroki§, Urban Deutsch¶, Yin-Shan Ng†§, Anthony P. Adamis§||, and Patricia A. D'Amore†§**

From the †Program in Biological and Biomedical Sciences and the **Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, the §Laboratory for Surgical Research, Children's Hospital, Boston, Massachusetts 02115, the ||Department of Ophthalmology, Harvard Medical School/Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02115, and the ¶Max-Planck Institut für physiologische und klinische Forschung, Bad Nauheim, Germany

We describe the genomic organization and functional characterization of the mouse gene encoding vascular endothelial growth factor (VEGF), a polypeptide implicated in embryonic vascular development and postnatal angiogenesis. The coding region for mouse VEGF is interrupted by seven introns and encompasses approximately 14 kilobases. Organization of exons suggests that, similar to the human VEGF gene, alternative splicing generates the 120-, 164-, and 188-amino acid isoforms, but does not predict a fourth VEGF isoform corresponding to human VEGF₂₀₆. Approximately 1.2 kilobases of 5'-flanking region have been sequenced, and primer extension analysis identified a single major transcription initiation site, notably lacking TATA or CCAT consensus sequences. The 5'-flanking region is sufficient to promote a 7-fold induction of basal transcription. The genomic region encoding the 3'-untranslated region was determined by Northern and nuclease mapping analysis. Investigation of mRNA sequences responsible for the rapid turnover of VEGF mRNA (mRNA half-life, <1 h) (Shima, D. T., Deutsch, U., and D'Amore, P. A. (1995) *FEBS Lett.* 370, 203-208) revealed that the 3'-untranslated region was sufficient to trigger the rapid turnover of a normally long-lived reporter mRNA *in vitro*. These data and reagents will allow the molecular and genetic analysis of mechanisms that control the developmental and pathological expression of VEGF.

The mediators of neovascularization comprise a diverse collection of growth stimulators and inhibitors that have been so designated because of their abilities to affect angiogenesis *in vivo* and/or endothelial cell proliferation *in vitro* (for review, see Ref. 1). Vascular endothelial growth factor (VEGF)¹ was initially identified based on its ability to stimulate vascular permeability (called VPF, for vascular permeability factor) and was subsequently demonstrated to be an endothelial cell-specific mitogen and angiogenic factor (2, 3). *In vivo*, VEGF expression has been correlated with embryonic, physiological, and pathological blood vessel growth (4-6). VEGF's role as a

mediator of angiogenesis has been confirmed in two distinct pathologies; VEGF has been demonstrated to be a necessary component of experimental tumor angiogenesis and tumor growth in rodents (7, 8), and, more recently, it has been shown to be causative in the development of ocular angiogenesis secondary to retinal ischemia (9, 10).

The spatial and temporal expression patterns of VEGF and its tyrosine kinase receptors, *flt-1* and *flk-1/KDR*, during periods of blood vessel growth have also led investigators to suggest a paracrine role for VEGF during the development of the embryonic vasculature (11, 12). The VEGF receptor *flk-1* is expressed in regions of the early mesoderm, which are presumed to give rise to angioblasts, and is currently the earliest known molecular marker for the endothelial cell lineage. During later stages of embryogenesis, *flt-1* and *flk-1* receptor mRNA are restricted to the endothelium of vascular cords and blood islands, with VEGF mRNA expressed in adjacent embryonic tissues (13). Proof of a role for VEGF in vessel development comes from recent studies in which VEGF receptors were deleted by targeted disruption. Mouse embryos, in which the *flk-1* receptor was deleted by targeted disruption, lacked blood islands and died between days 8.5 and 9.5. In these embryos, no organized blood vessels were observed and hematopoiesis was dramatically reduced (14). Mice, in which *flt-1* was mutated by targeted disruption, were able to form endothelial cells but unable to assemble them into normal vascular channels and thus died at mid-somite stages (15).

From these and other observations, VEGF emerges as a mediator of vasculogenic and angiogenic events associated with a wide range of biological events (16). Consistent with this concept, the local and systemic signals responsible for orchestrating the growth and regression of new blood vessels must ultimately regulate VEGF gene expression. Numerous effectors of VEGF gene expression have been identified, including cAMP, steroid hormones, protein kinase C agonists, polypeptide growth factors, oxygen, free radicals, glucose, cobalt, and iron. The potential mechanisms through which these agents modulate gene expression are varied, and include transcriptional regulation through AP-1, AP-2, steroid hormone receptors, p53, and NFκB, as well as post-transcriptional control of mRNA stability (17-21).²

To begin an analysis of the relevant mechanisms controlling the developmental and pathological expression of VEGF and to develop reagents for defining the role of VEGF in embryonic development using mouse molecular genetics, we have isolated and characterized the mouse VEGF gene. The structure of the

* This work was supported by Grant EY05985 (to P. A. D.), Massachusetts Lion's Eye Research Fund (to A. P. A.), and Research to Prevent Blindness (to A. P. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U41383.

¹ The abbreviations used are: VEGF, vascular endothelial growth factor; kb, kilobase(s); PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair(s); UTR, untranslated region; LTR, long terminal repeat.

² M. Kuroki, E. E. Voest, L. V. Beerepoot, M. Tolentino, R. Y. Kim, K. A. Colby, K.-T. Yeo, and A. P. Adamis, submitted for publication.

gene was determined by restriction mapping, sequencing of intron-exon junctions, definition of the transcription initiation and termination sites, and analysis of the sequence representing the VEGF proximal promoter. Using these structural data, we have assayed the mouse VEGF gene for cis-regions responsible for different aspects of gene regulation and to this end describe gene segments sufficient to promote basal transcriptional activity and post-transcriptional regulation of the VEGF gene.

MATERIALS AND METHODS

Gene Isolation and Physical Mapping—A 129 strain mouse cosmid genomic library prepared from liver (Stratagene) was screened with random prime ^{32}P -labeled probes corresponding to the mouse VEGF₁₆₄ coding region (kindly provided by Dr. Kevin Claffey, Beth Israel Hospital, Boston). Approximately 5×10^5 colonies were screened on nylon filters (Hybond N, Amersham) by hybridizing the VEGF probe overnight at 65 °C in 500 mM phosphate, pH 7.2, 7% SDS, 1% bovine serum albumin, 1 mM EDTA, and 100 $\mu\text{g}/\text{ml}$ sheared, denatured salmon sperm DNA. Several positive clones were identified in this initial screen. After two rounds of rescreening and colony purification, cosmid DNA was isolated and analyzed by restriction digestion and Southern blot to identify useful DNA fragments for subcloning. Restriction-digested cosmid insert DNAs were subcloned into pBSII (Stratagene) for further analysis. Nucleotide sequencing of subclones indicated that VEGF cosmid clones encompassed the 3'-half of the VEGF gene (beginning near exon 4) and extended 30–40 kb in the 3'-direction.

To isolate additional clones encompassing the 5'-end of VEGF, a 280-bp cDNA probe template spanning exons 1–3 was generated, using the polymerase chain reaction, and used to rescreen 5×10^5 colonies. No additional VEGF clones were identified. As an alternative, the exon 1–3 probe was used to screen a 129 mouse genomic library in the lambda vector EMBL3 (kindly provided by Dr. Richard Moss, Brigham and Women's Hospital, Boston). The library was screened on charged nylon membranes (GeneScreen Plus, DuPont NEN) by hybridization at 42 °C in $5 \times \text{SSPE}$, 50% deionized formamide, $5 \times \text{Denhardt's}$, 10% dextran sulfate, 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ sheared, denatured salmon sperm DNA. Two positive clones were identified from screening 1×10^6 plaques. After two rounds of plaque purification, phage DNA was isolated and analyzed further. Restriction digestion and Southern blot analysis of genomic clones indicated that they encompassed the 5'-end of the VEGF gene; one clone, designated lambda 8, overlapped with cos15, a cosmid clone that terminates in the intron upstream of the exon 4 sequence. Sequence data, restriction maps, and Southern blot analysis of the mouse VEGF gene were compiled from 9- and 7.5-kb *EcoRI* subclones that encompass the VEGF coding region (see Fig. 1).

DNA Sequencing—Genomic fragments cloned into pBSII were sequenced with vector and gene-specific primers using the Sequenase 2.0 kit (U. S. Biochemical Corp.). Exon-specific primers (kindly provided by Dr. Greg Robinson, Hybrion Inc., Worcester, MA) were synthesized, based on alignment of the mouse VEGF cDNA sequence, with the published human VEGF exon structure (22). Nucleotide sequence analysis was performed with MacDNAsis 3.4 software (Hitachi).

Southern Blot Analysis—Genomic DNA was prepared from 129 mouse spleen using standard protocols (23). Restriction enzyme-digested DNA (10 μg) was electrophoresed in 0.7% TBE-agarose gels overnight. Following electrophoresis, DNA was depurinated in 0.25 M HCl, denatured, and transferred to charged nylon (GeneScreen Plus) in 0.4 M NaOH. Membranes were rinsed in $2 \times \text{SSPE}$, dried, and hybridized as described for phage library screening. The probe was a 700-bp *SmaI*-*BglI* genomic DNA fragment spanning sequences in intron 3 through intron 5 (see Fig. 1, probe A). After high stringency washes, hybrids were visualized by autoradiography.

Transcript Mapping—Total RNA isolation was performed using a modification of the acid guanidinium-phenol-chloroform extraction protocol (24) with RNazol B (Tel-test). Total RNA was further divided into poly(A)⁺ and poly(A)[−] pools, using oligo(dT) affinity chromatography (25). For primer extension, an antisense oligonucleotide primer (5'-CTGGTGAAGTCCTGATAGTCTGCCTTGTG-3') was designed to hybridize to sequences approximately 100 bp downstream from the predicted initiation site. Primer selection and estimates of the location of the transcription initiation site were obtained by aligning mouse VEGF nucleotide sequence with the published sequence for the human VEGF 5'-untranslated region (UTR) and proximal promoter region (22). ^{32}P -End-labeled probes were separately hybridized to poly(A)⁺ and poly(A)[−] RNA obtained from normoxic or hypoxic cultures of C1271 mouse mam-

mary epithelial cells (ATCC) (26) in 50 mM Tris, pH 8.3, 50 mM KCl, 10 mM MgCl_2 . Primer extension was initiated by the addition of 1 mM deoxynucleotides, 10 mM dithiothreitol, and 1 unit of avian myeloblastosis virus reverse transcriptase in a final volume of 26 μl followed by incubation of the mixture at 42 °C for 30 min. Products were separated on 8 M urea, 6% polyacrylamide gels and visualized by autoradiography. The precise location of the transcription initiation site was determined by comparing the migration of the major reverse transcription product with a dideoxy sequence ladder generated with the same oligonucleotide used for primer extension.

To identify the 3'-end of VEGF transcripts, a series of adjacent ^{32}P -labeled genomic DNA probes (probe A, 1.3-kb *EcoRI* fragment; probe B, 750-bp *EcoRI*-*SfiI* fragment; probe C, 600-bp *SfiI* fragment; see Fig. 7), spanning the 3'-end of the VEGF genomic clone, were hybridized to immobilized mouse lung RNA, according to standard protocols. Because of a weak, yet significant, hybridization signal, probe C was predicted to overlap regions of genomic DNA encoding the VEGF transcript and the 3'-flanking sequence. A nuclease protection assay was used to more precisely define the 3'-end. A 4.4-kb *SmaI* genomic DNA fragment (spanning the 3'-UTR and flanking region of VEGF) cloned in pBSII (Stratagene) was used for riboprobe synthesis. The DNA template was linearized at an *NcoI* site within the probe B region, and an antisense RNA probe of 2.2 kb (see Fig. 7) was transcribed with T7 RNA polymerase according to standard protocols (27). The probe was hybridized to C1271 mouse mammary epithelial cell total RNA or yeast tRNA overnight at 30 °C in 40 mM PIPES, pH 6.4, 400 mM NaCl, 80% deionized formamide, 1 mM EDTA. The mixture was diluted in nuclease digestion buffer (50 mM sodium acetate, pH 5.0, 30 mM NaCl, 1 mM zinc acetate, 20 $\mu\text{g}/\text{ml}$ denatured calf thymus DNA) and then incubated in the presence of 300 units of mung bean nuclease (Life Technologies, Inc.) for 1 h at 30 °C. Reaction products were separated by denaturing gel electrophoresis in an 8 M urea, 4% polyacrylamide gel and visualized by autoradiography.

Mouse VEGF Promoter-Luciferase Constructs—A 1.6-kb fragment of VEGF genomic DNA, which encompasses 1.2 kb of the 5'-flanking sequence, the transcription start site, and 0.4-kb of corresponding 5'-UTR, was ligated upstream of a promoterless luciferase gene in the pGL2-basic plasmid (Promega). 5'-Deletions were made using convenient restriction sites within the VEGF promoter and the pGL2 multiple cloning site. These included a 445-bp deletion from the 5' terminus of the 1.6-kb fragment (−1217 bp) to the *Apal* site at −772 bp, a 768-bp deletion from the 5' terminus to an internal *Mlu* site at −449 bp, and a 1.3-kb deletion from −1217 bp to the *SmaI* site at +126 bp (see Fig. 6). The promoterless plasmid, pGL2-basic, and a construct in which the promoter was cloned into pGL2-basic in opposite orientation with respect to transcription initiation served as negative controls. The human cytomegalovirus immediate early gene promoter/enhancer region was fused to the luciferase reporter for use as a positive control.

DNA Transient Transfection, Luciferase, and Alkaline Phosphatase Assays—Transient transfections were performed with 4 μg of test plasmid using Lipofectin (Life Technologies, Inc.) reagent according to the manufacturer's protocol. As a control for transfection efficiency, pRcCMVAP (4 μg), encoding a secreted placental alkaline phosphatase gene (kindly provided by Dr. Gerhard Raab, Children's Hospital, Boston), was cotransfected with test plasmids. Alkaline phosphatase activity was determined (28) and used to normalize luciferase assay values. Luciferase activity in cell extracts was assayed 48 h post-transfection according to standard protocols (27) using a Bioscan luminometer (Wallace).

neo/VEGF Fusion Constructs, Generation of Stable Cell Lines, and mRNA Stability Assays—A 4.4-kb *SmaI* fragment, encompassing 2 kb of the VEGF 3'-UTR, including poly(A) signals, and 2.4 kb of 3'-flanking sequence, was ligated into the *SmaI* site downstream of a neomycin resistance gene (*neo*) open reading frame in the eukaryotic expression plasmid LTR-*neo* (kindly provided by Dr. Michael Cole, Princeton University, Princeton, NJ). LTR-*neo* transcripts terminate with SV40 UTR and poly(A) sequences (29). LTR-*neo* and LTR-VEGF constructs were individually transfected by electroporation into C1271, a mammary epithelial cell line. The plasmid pPGKhyg, encoding the hygromycin resistance gene, was cotransfected with test plasmids to allow selection of stable transfectants with hygromycin (200 $\mu\text{g}/\text{ml}$). Stable colonies (50–100) were pooled and expanded for use in mRNA stability assays.

Actinomycin D chase assays and Northern blot analysis of total RNA were performed as described (20). Briefly, confluent cells were incubated in culture media containing actinomycin D (5 $\mu\text{g}/\text{ml}$) and subsequently incubated for 0–8 h in standard culture conditions. Total RNA was extracted by the modified acid-phenol method using RNazol B (Tel-test) and analyzed by Northern blot for LTR-*neo*, LTR-VEGF, and

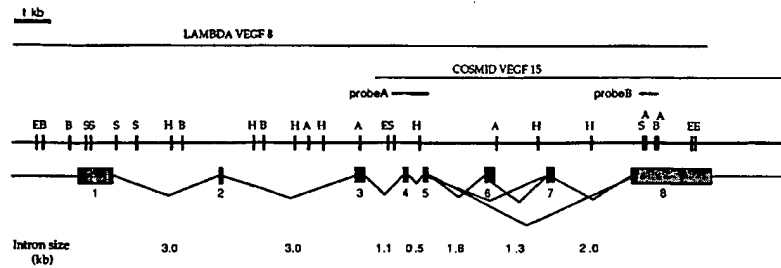


FIG. 1. Genomic organization of mouse VEGF. Restriction map of the mouse VEGF gene and flanking regions as established from the lambda 8 and cosmid 15 clones. Sites are marked for the enzymes *Acl* (A), *Bam*HI (B), *Eco*RI (E), *Hind*III (H), and *Sma*I (S). Locations of exons 1–8 and intron sizes are indicated. The VEGF open reading frame is indicated by black shading.

β -actin mRNA levels. 15 μ g of RNA/sample were fractionated by denaturing gel electrophoresis and capillary blotted to charged nylon (GeneScreen Plus). Prehybridization and hybridization were carried out in $6 \times$ SSPE, $5 \times$ Denhardt's, 50% formamide, 1% SDS, and 100 μ g/ml sheared, denatured salmon sperm DNA. Probes for Northern blot analysis were random prime labeled with 32 P, using the following templates: a 280-bp fragment encoding exons 1–3 of the mouse VEGF cDNA open reading frame, a 440-bp *Bss*III–*Sma*I fragment of the *neo* gene, and a 400-bp fragment from the 3'-UTR of human β -actin (30).

RESULTS

Isolation and Preliminary Characterization of the Mouse VEGF Gene—To isolate the VEGF gene, a probe representing the mouse VEGF₁₆₄ open reading frame was used to screen a cosmid genomic DNA library prepared from strain 129 mouse liver. From an initial screen of 5×10^5 colonies, several positive clones were identified and analyzed further. Sequencing of the termini of cosmid inserts indicated that all of the clones originated within the middle of the VEGF coding region and proceeded approximately 30–40 kb in the 3' direction. Rescreening the cosmid library with the same probe, or a probe corresponding to the 5'-half of the VEGF open reading frame, did not identify cosmids harboring the 5'-end of the VEGF gene. Because the 5'-end of VEGF did not appear to be represented in the cosmid library, a 129 mouse genomic DNA library constructed using the EMBL3 lambda phage vector was screened. Using a probe spanning exons 1–3 to screen 1×10^6 plaques, two clones spanning the 5'-end of the VEGF gene were identified. One of these clones, designated lambda 8, also overlapped with regions represented in a VEGF cosmid clone (cos15); therefore, these two clones were used for structural analysis of the VEGF gene. The relation of the phage and cosmid clones to the mouse VEGF gene are shown schematically in Fig. 1.

A restriction map for the two overlapping clones was assembled by single, double, and partial digestions with *Eco*RI, *Bam*HI, *Acl*I, *Hind*III, and *Sma*I restriction enzymes (Fig. 1). The locations of exons relative to the restriction map were established by nucleotide sequencing the restriction sites proximal to exons and Southern blot analysis of cloned DNA with exon-specific probes (data not shown). Restriction enzyme analysis of genomic DNA by Southern blot was used to confirm mapping data and verified that mouse VEGF was encoded as a single copy gene (Fig. 2). The overlapping genomic clones define a contiguous stretch of 45 kb of DNA, of which approximately 14 kb represents the mouse VEGF coding region.

Exon-Intron Nucleotide Sequence and Organization—To determine the genomic organization of the mouse VEGF gene, nucleotide sequences of the coding regions and intron-exon borders were determined (Fig. 3) and aligned with published mouse VEGF cDNA sequences (4). Similar to the human gene, the coding region of mouse VEGF is interrupted by seven introns, with exons 1 and 8 containing relatively short segments of the coding region and the UTRs (22). The organization

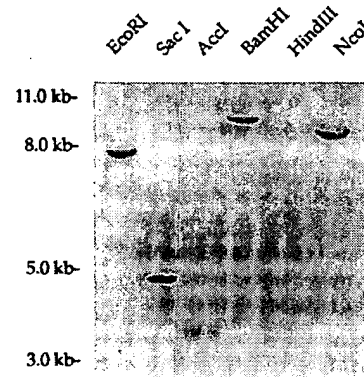


FIG. 2. Genomic Southern blot analysis of mouse VEGF. 129 mouse genomic DNA (10 μ g) was digested with the indicated enzymes and analyzed in a Southern blot using a mouse VEGF genomic DNA probe (probe A as illustrated in Fig. 1). The hybridizing fragment in *Hind*III-digested DNA is less than 3.0 kb in size and has been run from the gel.

of the mouse coding region suggests that the three VEGF protein isoforms, VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈, are created by alternative splicing of a single gene. The VEGF₁₈₈ splice variant includes all 8 exons, whereas the removal of exon 6 or both exons 6 and 7 yields the VEGF₁₆₄ and VEGF₁₂₀ splice variants, respectively. The mouse VEGF gene structure is shown schematically in Fig. 1.

A fourth human VEGF isoform, designated VEGF₂₀₆, was previously identified by polymerase chain reaction amplification of VEGF isoforms from a fetal human liver cDNA library (31). From a comparison of cDNA and genomic sequence, this splice variant was predicted to be derived from the utilization of an alternative splice donor site downstream from the site originally identified for exon 6. The mouse gene would not be predicted to encode an isoform homologous to human VEGF₂₀₆. When compared to the published human sequence, the sequence of the corresponding region of the mouse gene contains an additional nucleotide, creating a frameshift that results in an in-frame stop codon (Fig. 4).

Functional Analysis of the Mouse VEGF Promoter—Alignment of sequences from the 5'-regions of mouse and human VEGF and primer extension analysis were used to map the site of transcriptional initiation for VEGF mRNA in the C127I mouse mammary epithelial cell line. Low levels of VEGF mRNA were detectable in the C127I cells grown under typical culture conditions, but were dramatically induced by exposing the cells to a low oxygen environment (Fig. 5A). An antisense primer, predicted to anneal approximately 100 nucleotides downstream of the transcription initiation region, was chosen based on alignment of the human proximal promoter region with corresponding mouse sequence. The primer was annealed to poly(A)⁺ RNA isolated from normoxic and hypoxic cultures

-1217 t g t t a g a a g a t g a a c c g t a a g c c t a g g c t a g a a c t g a g g g a g c c t a c t a c t c c c a c c c t t c c g a g g t t
-1147 g g c g g a g g a c t g g g c a g c t g g c c a c t a c c t t t c t g a t g c t a g g g t a g g t t g a t a c c a c t g c c g
-1077 c c t g g c c g c t t c t g c c c c a t t g c a c c c t g g c t c a g t t c c c t g g c a a c a t c t c t g t g t g t g t g
-1007 t g t g t a g a g a g a g a t c a g g a g a a c a g g c c t g t c t g c c a g a g t g t c t c c c t c a g g g c t
-937 c t g c a g a c t a c a c a g t g c a t a c g t g g g t t t c c a c a g g t c g t c t c a c t c c c g c a g t a a c t c a g
-867 a a c c a c c c c g t t c t c a g t g c c a a a t t t g g t g c a a t t c t c c a g a g a g c t c t c t g g a a c t
-797 t c c c a g a g a t c c c a t t c a c c c a g g c c c t a g c t c c t g a t g a c t g c a g a t c a g a c a a g g c t c a g a t a a
-727 g c a t a c c c c c c c c c c c c c c c c c c c a t a t a a a c c a c a g t t a t g c t t c c g a g g t c a a a c a c
-657 g c a a c t t t t g g g t g t g t g t a t g t c a g a a c a c g c a a t t a t t g g a g c t c a a a g t c t g c g c a c t a c
-587 a g a t c a t c t c t a c c c c c t t c c a a g a c c c g t g c a t t t g a g c a a g a g t t g g g t g t g a t a a t g a g t
-517 c a c t a g g g g g c t c g g c a t c a c g g a g a t c t a a c t t g g c a g c g a g t c t g c t g a g g a g a a c g
-447 c g t g t t c a a t g t g a g t g c t g c t g t g t g t g t g t g t g t g t g a g g t g g g a g a a a g c
-377 c a g g g t c a c t c a g t g t c c c a t c c a c t c c t g c a g c t c c g c c t c c a a c c c c a c t t t c
-307 t c c a t a t c c t g g a a g g a a t g t c t t a g a c c t g t c c g a t a t a a c c t a c t c t c c t g t c t c c c t g
-237 a t t c c a a t a c t c t g g g a t t c c a g t g t g t c c t g a g c c a t t g a a g g g t g c a c a g a t a a t t t g a g g
-167 c c t g g a c c t g g t a a g g g t t a g e t t t c a t t t c g e g g t a g t g c c t a g g g c t c c c g a a a g g c g g t
-97 g c c t g g c t c a c c a g a c c g t c c c c g g g c g g g t c t g g g g t g g a g c t a g a t t t c c t t t
-27 t t c t t c c a c c g c t g t t a c c g t g a g t g c g a g a g g c t t g g g c a g c c a g c t g c a g c a g c g c g c g g c
44 a c t g g g g g c a g c t g a g c g g g c a g c g a g c t g t g c g a g c g a g c a g c a a g g c a g a c t a t c a g c g
114 g a c t c a c a g c c c g g a g t g t g t c t g g a t t g a t t c a a a c c t t a a t t t t t t t t t t a a a c t
184 g t a t g t t t a c g c t t a a t t a t t t t t g c t c c a t t c c c c t t a a a t c g t g c a a c g g t t t g a g a g
254 g t g g t t c t a c t c c c t a a a t a c t t g a t t g t g g a a t c a g c a g a g a a g a g g t a t c a a g a g c t c
324 c a g a g a a a g t c a a g a a g a g a g a g a c c g g t c a g a g a g c g c g t g g c g a c g a c a g a g a g g
394 g a c a g g g c a a a g t g a c t t g a c t t g t t t t g g g g t g a c c g c a g a g c g c g g c t g a c c t c c c c t t c
464 g a t c t g a t c g g a c a g t c g c t g c g a g a c a g a c a g a c a c c c c c a g c c c a g c c a c c a c c t
534 c c t c g c g c g g g c t g c g a c g g t g g a c g c g g c g c g a g a a c c g a a g c c c g c c g a g g c g g
604 g t g g a g g g g t c g g g c t c g g g a t t g c a c g a a a c t t t t g t c a a c t t c t g g g c t t t t c t g c t c o g
674 t a g t a g c c t g t g t g c g c g c a g g a c a a a c c g a t c c g a g c t g g g a g a a g c t a g c t g g c c c t g g a
744 g a g g c g g g c c g a g a g a g g g g a g g a a g a g a g a g g g g c a c a g t g g g c t g g c t c o g
814 g g a g c g a g c t c a t g a c g g t g a g g c g c g t g t g c g a c a g t g c t c c a g c c g c g c g c c c a g
884 g c c c g c c c g g c c t g g t t c a g a a g g a g a g g c c c a a g g c g c g a a g a g a g c g g g t g c c t c
954 g a g t c c g a g c c g g a g a g a g g a g c g a g c g c g c g c c c c c g c c c c a g c c t c c g a a a c t g t a c t t
..... EXON 1 GCCAAGtaagcggctgtgccgtctgctgt..... tct
ttaetctttgtttgttctcagTGATCC..... EXON 2 ATGAAGtgagtcctatgtctttaa
ggatccctgtt..... ctggtgtccctccacacagTGATCA..... EXON 3 CATG
CAGgtggacactgcggcacaggggacggg..... atctgtccctccctctacagATCATG
..... EXON 4 ATGCAGtgagccagacttcacactcagg..... cctttctctc
cccactgcagAACAA..... EXON 5 AGAAAgttaagtggccactgtctagcattt.....
..... tttctgttttatattttccagAAATC..... EXON 6 GAGCCTgtacgttgggtgcc
ctgctgtcactcctgt..... tttcttttgccttttgcagTCATGT..... EXON 7 ..
..... TTGCAGgttggtttccagaggggcaagca..... tctcttgcactttcccatagATG
TCA..... EXON 8

FIG. 3. Nucleotide sequence for mouse VEGF intron-exon borders and the sequence surrounding the mouse VEGF transcription initiation site. Intron and UTR sequence is shown in lower case letters, coding sequence is shown in upper case letters, and the VEGF translation start and stop codons are boxed. An arrow indicates the initiation site of RNA synthesis and is designated +1. Consensus binding sites for relevant transcription factors are marked as follows: AP-1, thin line; AP-2, heavy hatched line; NFkB, broken line; Sp1, heavy line.

mouse intron/exon 6

TAC GTT GGT GCC GCT GCT GTC TAA TTC CTT GGA GCC TTC CTG GTC...
Y V G A A A V *

human intron/exon 6

TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG AGC CTC CCT GGC...
Y V G A R C C L M P W S L P G...

FIG. 4. Comparison of mouse exon-intron 6 with the corresponding region of human VEGF. The nucleotide insertion in the human sequence that creates a continuous open reading frame in VEGF₂₀₆ is marked. The translation stop codon present in the homologous region of mouse VEGF genomic DNA is designated with an asterisk. Predicted amino acids are indicated below the nucleotide sequence using a single letter format.

of C1271. Primer extension using poly(A)⁻ RNA was used as a negative control. After reverse transcription and denaturing gel electrophoresis, a single abundant primer extension product of 123 bp was detected in the mRNA from hypoxic cell cultures (Fig. 5B). As would be expected, significantly lower levels of reaction product were seen in mRNA from normoxic cultures or in poly(A)⁻ RNA from normoxic and hypoxic cultures. The precise location of transcription initiation was determined by comparison of the migration of the extension prod-

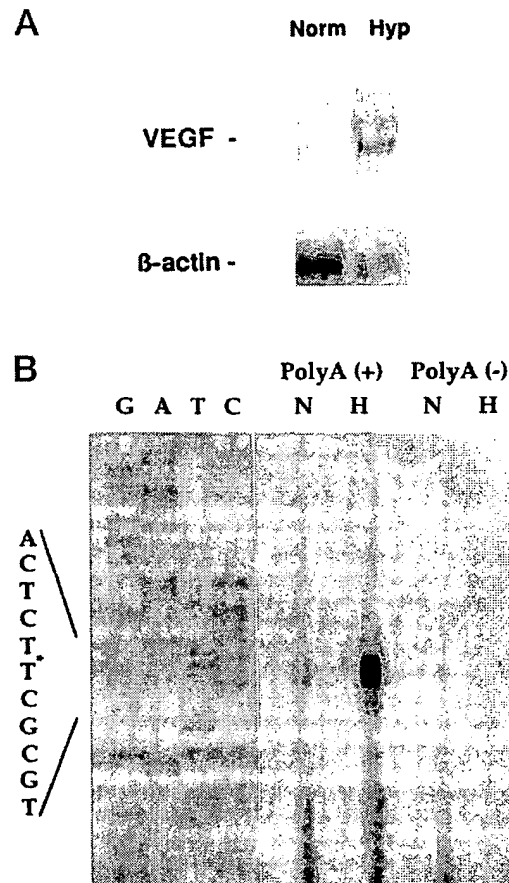


FIG. 5. Primer extension analysis of the VEGF transcription start site. A, Northern blot analysis of total RNA from normoxic and hypoxic cultures of C1271 demonstrating the differences in steady-state VEGF mRNA levels. B, primer extension products were separated by electrophoresis on a denaturing polyacrylamide gel along with a DNA sequencing ladder generated with the same oligonucleotide used for primer extension. Poly(A)⁺ and poly(A)⁻ RNA from normoxic (N) or hypoxic (H) cultures of C1271 mouse mammary cells were used in the reactions. A single primer extension product was detected in the poly(A)⁺ RNA, indicating the location of the transcriptional start site (the complementary nucleotide is shown by an asterisk).

uct with a sequencing gel ladder generated using the same primer and is designated +1. Nucleotide sequence is shown for regions upstream of the mouse VEGF open reading frame including the 1.0-kb 5'-UTR and 1.2 kb of the proximal promoter region (Fig. 3).

To determine if the sequences upstream of the transcription initiation site are sufficient to direct transcription, a 1.6-kb fragment, including 1.2 kb of 5'-flanking region and 0.4 kb of 5'-UTR, was fused in both orientations to a promoterless luciferase transcription reporter gene and examined for the ability to mediate basal transcription. In addition, 5'-deletions in the putative promoter region were also monitored for their effect on reporter activity.

The murine astrocytoma cell line, C6, was transiently transfected with reporter constructs, and cell extracts were assayed for luciferase activity 48 h post-transfection. VEGF sequences fused to the reporter in the appropriate transcriptional orientation consistently produced a 7-fold increase in luciferase activity when compared to a promoterless luciferase construct (Fig. 6). In contrast, VEGF sequences fused in the opposite transcriptional orientation did not induce a significant level of reporter activity. Deletion of 445 or 770 bp from the 5'-end of

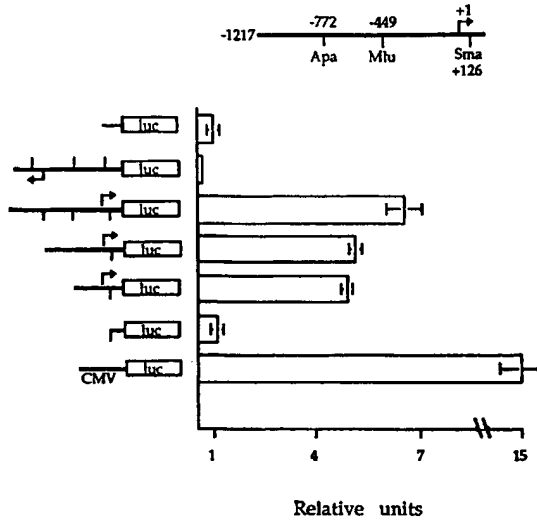


FIG. 6. Functional analysis of the mouse VEGF promoter. Relative luciferase activities were obtained from transiently transfected VEGF promoter-luciferase constructs in C6 glioma cells. Schematic representation of assay constructs (see "Materials and Methods") is shown. All luciferase values were normalized for transfection efficiency, using placental alkaline phosphatase activity, and are expressed as the level of luciferase activity relative to the activity of the promoterless luciferase control plasmid pGL2 basic. Data shown are from duplicate analyses and are representative of five separate experiments. Within these five experiments, peak VEGF promoter activity varied from 7 to 10-fold relative to negative controls.

the promoter fragment resulted in a 25% decrease in reporter activity, whereas a 1.3-kb deletion, which removed putative promoter sequences and the transcriptional initiation site, reduced luciferase activity to background levels.

Identification of the VEGF Transcription Termination Region—The region of transcriptional termination for VEGF was mapped by RNA hybridization and nuclease protection assays. First, to approximate the junction of the VEGF 3'-UTR and the 3'-flanking region, genomic probes spanning the putative 3'-region of the VEGF gene were used in a slot blot hybridization analysis of mouse VEGF RNA (Fig. 7A). Independent hybridization of all three probes to the mouse RNA resulted in detectable, though variable, signal (Fig. 7B). No signal was detected in hybridizations to a yeast tRNA negative control. Relative to probes A and B, probe C weakly hybridized to immobilized mouse RNA. From the dramatic decrease in hybridization signal seen for probe C, it was predicted that this region of DNA spanned the junction of VEGF transcriptional termination and the non-transcribed 3'-flanking region.

Nuclease protection analysis of mRNA from C127I cells was used to obtain more precise information on the 3'-end of VEGF transcripts. A 2.2-kb radiolabeled antisense riboprobe was generated from a genomic DNA template and hybridized to total RNA from hypoxic and normoxic cultures of C127I and a yeast tRNA negative control. Following nuclease digestion and electrophoretic separation of nuclease products, a 510–520-nucleotide nuclease-resistant species was observed in hypoxic RNA hybrids (Fig. 7C). The same protected fragment was observed from normoxic RNA hybrids after extended exposures of the analytical gel, reflecting the 10–20-fold differences in VEGF mRNA levels between normoxic and hypoxic cultures. The faint band of about 450 bp may reflect the existence of a less utilized alternative termination site. No product was seen in the yeast tRNA negative control. These data place the site of transcriptional termination approximately 510 bp downstream from the riboprobe terminus, leading to a 3'-UTR of approximately 2.2 kb.

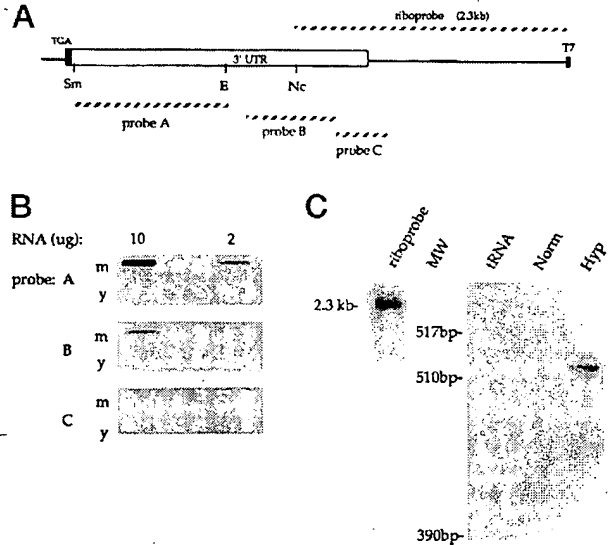


FIG. 7. Analysis of the mouse VEGF 3'-end. A, schematic of exon 8 and corresponding flanking region. *EcoRI* (E), *SmaI* (S), and *NcoI* (Nc) restriction sites are marked. Regions corresponding to 3'-UTR probes A, B, and C and the T7-synthesized riboprobe are indicated. B, slot-blot hybridization analysis of 10 or 2 µg of mouse total RNA (m) and control yeast tRNA (y) with 3'-UTR probes. C, analysis of mung bean nuclease-digested C127I RNA/riboprobe hybrids. A 510–520-nucleotide digestion-resistant product is present solely in total RNA from hypoxic C127I cultures.

Identification of VEGF mRNA Destabilizing Sequences—We have previously demonstrated that VEGF mRNA from cells grown under normoxic conditions is highly unstable, with a half-life of <1 h (20). Whereas the average half-life of cellular mRNAs is approximately 8 h, the mRNAs for many growth factors and oncoproteins (e.g. granulocyte-macrophage colony stimulating factor, *c-fos*, *c-myc*) are unstable, with half-lives ranging from 20 to 60 min. The signals responsible for destabilization of these mRNAs are most frequently located in the 3'-UTR (29, 32).

To investigate if a determinant of mRNA destabilization is present in the VEGF 3'-UTR, genomic sequences corresponding to this region, including the putative polyadenylation signal, were fused to a neomycin (*neo*) reporter mRNA (designated LTR-VEGF). The VEGF 3'-UTR replaces SV40 DNA sequences that normally terminate the *neo* transcripts. The *neo*/SV40 mRNA fusion (designated LTR-*neo*) is normally quite stable, with a half-life of >8 h. The addition of destabilization sequences from the 3' UTR of granulocyte-macrophage colony stimulating factor, *c-fos*, and *c-myc* to LTR-*neo* mRNA has been shown to direct its rapid decay (29).

Actinomycin D chase studies were used to compare the rate of decay for LTR-VEGF mRNA to that of LTR-*neo* mRNA (control) in the C127I mammary epithelial cell line. As expected, the control LTR-*neo* mRNA remained stable throughout an 8-h period in the absence of transcription (Fig. 8). In contrast, LTR-VEGF fusion mRNA behaved similar to the endogenous VEGF mRNA, with both VEGF and LTR-VEGF transfectants undergoing rapid decay with half-lives of less than 1 h. Levels of endogenous β -actin mRNA, a transcript with an average half-life, were relatively stable under each experimental condition over the time course of the experiment (33).

DISCUSSION

VEGF has been implicated as a multi-functional effector of vascular cell function. In addition to its well documented angiogenic properties, VEGF is also a potent stimulator of leuko-

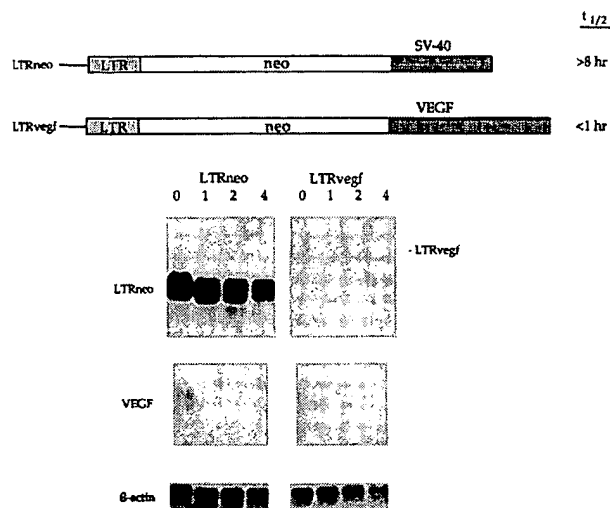


FIG. 8. The VEGF 3'-UTR contains a region that promotes destabilization of a normally stable fusion mRNA. LTR-*neo* and LTR-VEGF transfectants (schematic of fusion mRNA constructs shown; see "Materials and Methods") were used to analyze *neo* fusion mRNA, VEGF mRNA, and β -actin mRNA decay using an actinomycin D chase protocol. Time (h) after the addition of actinomycin D is marked above each lane.

cyte migration, vascular permeability, and procoagulant functions in endothelium (34, 35). Moreover, the presence of significant VEGF mRNA and protein in various tissues of the adult suggests an additional role for VEGF in the maintenance of normal vascular cell integrity and/or behavior (4). How VEGF gene expression is regulated during the transition from periods of vascular quiescence to active vascular growth, remodeling, and repair is not understood. To begin to investigate the structure-function relationships critical for the regulation of VEGF expression, we have characterized the mouse VEGF gene.

Mapping the VEGF Transcriptional Unit—We have isolated genomic clones encompassing the VEGF gene, as well as 1.2 kb of 5'-flanking sequence and more than 25 kb of 3'-flanking sequence. Similar to the human gene, the mouse VEGF coding region is distributed across 8 exons, spanning 14 kb of genomic sequence. Exons 1 and 8 contain the translation start and stop codons, respectively, and the extensive UTR regions. Sequence data and primer extension analysis of the transcription initiation site indicate that the mouse VEGF gene, like the human, has an unusually large 5'-UTR of approximately 1.0 kb (22). The site of VEGF transcription termination had not been previously defined for any of the VEGF genes; therefore, the size of the mouse 3'-UTR and approximate site of poly(A) addition were determined. Results from nuclease protection analysis revealed that the site of VEGF transcriptional termination is approximately 2.2 kb downstream from the end of the VEGF open reading frame. Including a 1.0-kb 5'-UTR, a coding region of 0.5–0.6 kb, and a 3'-UTR of 2.2 kb, the size of a VEGF transcript predicted by mapping data would be 3.7–3.9 kb, a size corresponding to the sizes of the most commonly observed VEGF transcripts (36).

Genomic Organization of VEGF Splice Variants—Details of the exon-intron structure for mouse VEGF suggest that alternative splicing generates the mRNAs that encode the 120-, 164-, and 188-amino acid isoforms of VEGF similar to the human gene (22). Yet, unlike human VEGF, the sequence structure of mouse exon 6 does not support the existence of a mouse equivalent to the human VEGF₂₀₆ isoform. An in-frame stop codon is present in the mouse gene in the region corre-

sponding to the human VEGF₂₀₆ open reading frame. If synthesized and translated, mouse VEGF splicing variants that include this region would be predicted to produce a novel, secreted VEGF protein isoform of approximately 16 kDa. The existence of this mRNA or protein has not been described.

Since little is known about the physiological roles of the four VEGF isoforms, it is difficult to predict the functional significance of a divergence in isoform generation between humans and mice. The putative VEGF₂₀₆ isoform was identified using the polymerase chain reaction to amplify VEGF-related cDNAs and consists of an alternative splice variant with an extended exon 6 region that results in a 17-amino acid insertion relative to the VEGF₁₈₉ isoform. Analysis of VEGF synthesis, secretion, and bioactivity *in vitro* has revealed that an engineered VEGF₂₀₆ protein shares similar biochemical and functional properties with VEGF₁₈₉, suggesting that these two isoforms could provide redundant biological functions (31). To date, the biochemical and biological descriptions of VEGF₂₀₆ have been sparse and have relied on data obtained from the fusion of the N-terminal region of VEGF₁₆₅ to the partial VEGF₂₀₆ cDNA clone; the expression of native VEGF₂₀₆ mRNA or protein by tissue culture cells or *in vivo* has not been adequately described.

VEGF Promoter Analysis—Similar to other growth-related genes, including human VEGF, consensus TATA and CCAAT sequences for RNA polymerase II-initiated transcription are absent from the mouse VEGF core promoter region. Instead, GC-rich regions resembling consensus binding motifs for Sp1 are situated approximately 50–80 bp upstream of the predicted transcription start site in mouse VEGF. This organization is typical of the core region of many TATA-less promoters, which usually contain binding sites for sequence-specific transcriptional activators, frequently Sp1, and an initiator element (Inr) that overlaps the region of RNA synthesis (37, 38). The nucleotide sequence surrounding the VEGF mRNA initiation site, AGAAGCGCA (underline designates first transcribed nucleotide), does not conform to consensus Inr sequences; therefore, an investigation of VEGF transcriptional initiation is likely to shed light on a novel "TATA-less" mechanism for basal gene transcription (39).

Transient transfection assays indicate that a 1.2-kb segment of 5'-flanking region specifically directs the transcription of a reporter gene in VEGF-producing cells. Deletion of the 1.2-kb region, including the putative transcription initiation site, abolished promoter activity. Results from these functional analyses support transcript mapping and sequence analysis data that identify this DNA segment as the VEGF proximal promoter.

Transfection of C6 rat glioma with constructs deleting either 445 or 770 bp from the 5'-end of the 1.2-kb promoter region resulted in a similar 25% decrease in reporter activity, suggesting that cis-acting elements necessary for basal promoter activity in C6 cells reside in the 450-bp DNA segment deleted from the 5'-end of the promoter fragment. Yet, a relevant promoter activity resides within the first 450 bp upstream to the VEGF gene. Further studies will be required to identify and characterize the cis- and trans-acting components necessary for both basal and inducible regulation of VEGF gene transcription.

Sequence analysis of the 1.2-kb region, upstream of the transcription initiation site, revealed the presence of a number of potential cis-acting regulatory elements. Similar to the human VEGF gene, multiple consensus binding sites for AP-1 and AP-2 transactivating complexes are present. AP-1 activity has been shown to be stimulated by phorbol esters and growth factors, and both cAMP-dependent kinase and protein kinase C

pathways have been implicated in the activation of AP-2 (40, 41). Phorbol esters, peptide growth factors, and intracellular elevation of cAMP also induce steady-state VEGF mRNA, suggesting that the AP-1 and AP-2 consensus sites present in the VEGF promoter may mediate VEGF transcriptional activation in response to these effectors (17, 21). In addition, studies from a number of laboratories indicate that in some cells transcriptional activation plays a role in the up-regulation of VEGF mRNA by hypoxia (42–44). Further, the site of transcriptional initiation and numerous regions within the proximal promoter of mouse VEGF share significant similarity in both sequence and organization with the human homologue. The conserved organization of transcriptional regulatory sequences within the two promoters may imply a critical role for these regions in the proper regulation of VEGF gene expression. In contrast, consensus sites for NFkB, a transactivator implicated in the regulation of inflammation and stress response genes (45), are located 90 and 185 bp upstream of the initiation site in mouse VEGF, whereas NFkB consensus sites have not been identified in the human VEGF promoter.

Control of VEGF mRNA Stability—In addition to transcriptional control of gene expression, the level of mRNA for many oncoproteins and cytokines is regulated by post-transcriptional control of mRNA stability. For the best-studied of these genes, including granulocyte-macrophage colony stimulating factor and *c-myc*, the 3'-UTR is primarily responsible for the control of mRNA stability (29, 32). Results from our analysis of VEGF mRNA stability indicate that the VEGF 3'-UTR functions in an analogous fashion. Fusion of genomic DNA, representing the VEGF 3'-UTR, to a normally stable *neo* reporter gene destabilizes the transcripts, reducing the half-life from >8 h to less than 1 h. In contrast, LTR-*neo* and endogenous β -actin mRNAs were stable throughout the experimental time course, confirming that specific cellular mRNAs are targeted for destruction.

Experimental conditions that induce VEGF mRNA, such as phorbol ester treatment or hypoxia, are known to regulate mRNA stability (20, 32). Investigation of the cellular mechanisms controlling transcript stability suggest that certain mRNAs contain distinct structural elements for positive and negative regulation of mRNA stability. Specific destabilization sequences vary considerably but usually consist of AU-rich elements in the 3'-UTR of unstable mRNAs (46). Multiple AU-rich regions exist throughout the 3'-UTR of mouse VEGF (data not shown) and represent potential candidates for destabilizing sequences. Less is known about sequences that selectively or inducibly promote mRNA stability. For the transferrin receptor, a well studied model of inducible mRNA stability, the 3'-UTR contains a stem-loop sequence that interacts with inducible cellular factors to promote mRNA stability (47). During periods of iron starvation, RNA-protein interactions at the stem-loop sequence are dominant over the effects of a distinct region of AU-containing sequences, which otherwise trigger transferrin receptor mRNA degradation (48). Further investigation will be required to define sequences within the VEGF mRNA required for positive and negative regulation of post-transcriptional mRNA stability.

The findings reported here provide a framework for the comprehensive analysis of the regulation of VEGF expression and VEGF structure-function relationships. Such studies will be critical to understanding and eventually modulating the role of VEGF during physiological and pathological blood vessel growth.

REFERENCES

- Folkman, J. (1995) *Nature Med.* 1, 27–31
- Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) *Science* 246, 1306–1309
- Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) *Science* 219, 983–985
- Breier, G., Albrecht, U., Sterrer, S., and Risau, W. (1992) *Development* 114, 521–532
- Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H., and Keshet, E. (1993) *J. Clin. Invest.* 91, 2235–2243
- Miller, J. W., Adamis, A. P., Shima, D. T., D'Amore, P. A., Moulton, R. S., O'Reilly, M. S., Folkman, J., Dvorak, H. F., Brown, L. F., Berse, B., Yeo, T.-K., and Yeo, K.-T. (1994) *Am. J. Pathol.* 145, 574–584
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) *Nature* 362, 841–844
- Millauer, B., Shawver, L. K., Risau, W., and Ullrich, A. (1994) *Nature* 367, 576–579
- Adamis, A. P., Shima, D. T., Tolentino, M., Gragoudas, E., Ferrara, N., Folkman, J., D'Amore, P. A., and Miller, J. W. (1995) *Arch. Ophthalmol.*, in press
- Pierce, E. A., Avery, R. L., Foley, E. D., Aiello, L. P., and Smith, L. E. H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 905–909
- Millauer, B., Witzmann-Voss, S., Schnürch, H., Martinez, R., Møler, N. P. H., Risau, W., and Ullrich, A. (1993) *Cell* 72, 835–846
- Peters, K. G., De Vries, C., and Williams, L. T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 8915–8919
- Dumont, D. J., Fong, G.-H., Puri, M. C., Gradwohl, G., Alitalo, K., and Breitman, M. L. (1995) *Dev. Dynamics* 203, 80–92
- Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M., and Schuh, A. C. (1995) *Nature* 376, 62–64
- Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) *Nature* 376, 66–68
- Klagsbrun, M., and Soker, S. (1994) *Curr. Biol.* 3, 699–702
- Garrido, C., Saule, S., and Gospodarowicz, D. (1993) *Growth Factors* 8, 109–117
- Shweiki, D., Neeman, M., Itin, A., and Keshet, E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 768–772
- Goldberg, M. A., and Schneider, T. J. (1994) *J. Biol. Chem.* 269, 4355–4359
- Shima, D. T., Deutsch, U., and D'Amore, P. A. (1995) *FEBS Lett.* 370, 203–208
- Finkenzeller, G., Technau, A., and Marme, D. (1995) *Biochem. Biophys. Res. Commun.* 208, 432–439
- Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) *J. Biol. Chem.* 266, 11947–11954
- Herrmann, B. G., and Frischauf, A. (1987) in *Guide to Molecular Cloning Techniques* (Berger, S. L., and Kimmel, A. L., eds) Vol. 152, pp. 180–183. Academic Press, San Diego
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Shima, D. T., Adamis, A. P., Yeo, K.-T., Yeo, T.-K., Allende, R., Folkman, J., and D'Amore, P. A. (1995) *Mol. Med.* 1, 182–193
- Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (eds) (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
- Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. (1988) *Gene (Amst.)* 66, 1–10
- Schuler, G. D., and Cole, M. D. (1988) *Cell* 55, 1115–1122
- Hook, T. C., Newcomb, P. M., and Herman, I. M. (1991) *J. Cell Biol.* 112, 653–664
- Houck, K. A., Ferrara, N., Winer, J., and Cachianes, G. (1991) *Mol. Endocrinol.* 5, 1806–1814
- Shaw, G., and Kamen, R. (1986) *Cell* 46, 659–667
- Hargrove, J. L., and Schmidt, F. H. (1989) *FASEB J.* 3, 2360–2370
- Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) *Endocr. Rev.* 13, 18–32
- Senger, D. R., Van De Water, L., Brown, L. F., Nagy, J. A., Yeo, K.-T., Yeo, T.-K., Berse, B., Jackman, R. W., Dvorak, A. M., and Dvorak, H. F. (1993) *Cancer Met. Res.* 12, 303–324
- Adamis, A. P., Shima, D. T., Yeo, K.-T., Yeo, T.-K., Brown, L. F., Berse, B., D'Amore, P. A., and Folkman, J. (1993) *Biochem. Biophys. Res. Commun.* 193, 631–638
- Azizkhan, J., Jensen, D., Pierce, A., and Wade, M. (1993) *Crit. Rev. Eukaryotic Gene Expression* 3, 229–254
- Boisclair, Y., Brown, A., Casola, S., and Rechler, M. (1993) *J. Biol. Chem.* 268, 24892–24901
- Zawel, L., and Reinberg, D. (1992) *Curr. Opin. Cell Biol.* 4, 488–495
- Curran, T., and Franza, B. R. (1988) *Cell* 55, 395–397
- Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* 51, 251–260
- Minchenko, A., Salceda, S., Bauer, T., and Caro, J. (1994) *Cell. Mol. Biol. Res.* 40, 35–39
- Ikeda, E., Achen, M. G., Breier, G., and Risau, W. (1995) *J. Biol. Chem.* 270, 19761–19766
- Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995) *Circ. Res.* 77, 638–643
- Read, M. A., Whitley, M. Z., Williams, A. J., and Collins, T. (1994) *J. Exp. Med.* 179, 503–512
- Chen, C., and Shyu, A. (1994) *Mol. Cell. Biol.* 14, 8471–8482
- Koeller, D. M., Casey, D. M., Hentze, E. M., Chan, L.-N., Klausner, R. D., and Harford, J. B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 3574–3578
- Casey, J. L., Di Jeso, B., Rao, K., Klausner, R. D., and Harford, J. B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 1787–1791

The Human Gene for Vascular Endothelial Growth Factor

MULTIPLE PROTEIN FORMS ARE ENCODED THROUGH ALTERNATIVE EXON SPLICING*

(Received for publication, December 13, 1990)

Edmund Tischer, Richard Mitchell‡, Taymar Hartman, Maria Silva, Denis Gospodarowicz§, John C. Fiddes, and Judith A. Abraham¶

From California Biotechnology Inc., Mountain View, California 94043 and the §Cancer Research Institute, School of Medicine, University of California, San Francisco, California 94143

Vascular endothelial growth factor (VEGF) is an apparently endothelial cell-specific mitogen that is structurally related to platelet-derived growth factor. By Northern blot and protein analyses, we show that VEGF is produced by cultured vascular smooth muscle cells. Analysis of VEGF transcripts in these cells by polymerase chain reaction and cDNA cloning revealed three different forms of the VEGF coding region, as had been reported in HL60 cells. The three forms of the human VEGF protein chain predicted from these coding regions are 189, 165, and 121 amino acids in length. Comparison of cDNA nucleotide sequences with sequences derived from human VEGF genomic clones indicates that the VEGF gene is split among eight exons and that the various VEGF coding region forms arise from this gene by alternative splicing: the 165-amino acid form of the protein is missing the residues encoded by exon 6, whereas the 121-amino acid form is missing the residues encoded by exons 6 and 7. Analysis of the VEGF gene promoter region revealed a single major transcription start, which lies near a cluster of potential Sp1 factor binding sites. The promoter region also contains several potential binding sites for the transcription factors AP-1 and AP-2; consistent with the presence of these sites, Northern blot analysis demonstrated that the level of VEGF transcripts is elevated in cultured vascular smooth muscle cells after treatment with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate.

are active on a wide range of different cell types. In contrast, the recently identified vascular endothelial growth factor (VEGF) is a mitogen primarily for vascular endothelial cells; it appears to be inactive on fibroblasts, keratinocytes, vascular smooth muscle cells, lens epithelial cells, corneal endothelial cells, adrenal cortical cells, and granulosa cells (Gospodarowicz *et al.*, 1989; Ferrara and Henzel, 1989). Consistent with its endothelial cell mitogenicity, VEGF has also been shown to be angiogenic in the chick chorioallantoic membrane assay (Plouët *et al.*, 1989; Leung *et al.*, 1989).

VEGF was purified by Gospodarowicz *et al.* (1989) and Ferrara and Henzel (1989) from the conditioned medium of bovine pituitary folliculo stellate cells, utilizing an endothelial cell proliferation assay to monitor the biological activity of the protein. Use of a completely different assay, the stimulation of vascular permeability in guinea pig skin, resulted in the identification of a vascular permeability factor in tumor ascites fluid and in the conditioned medium from several tumor cell lines such as guinea pig line 10 hepatocarcinoma cells and the human histiocytic lymphoma cell line U-937 (Senger *et al.*, 1983, 1986; Connolly *et al.*, 1989a). Vascular permeability factor was subsequently found to be an angiogenic endothelial cell mitogen, and structural characterization by protein sequencing and cDNA cloning showed it to be identical to VEGF (Connolly *et al.*, 1989a, 1989b; Keck *et al.*, 1989). The range of cell types producing VEGF has now been extended to include a rat glioma cell line GS-9L (Conn *et al.*, 1990a), a mouse neuroblastoma cell line NB41 (Levy *et al.*, 1989), and the mouse pituitary cell line AtT-20 (Plouët *et al.*, 1989).

Purified VEGF is an approximately 46-kDa protein which dissociates upon reduction into two apparently identical 23-kDa subunits (Gospodarowicz *et al.*, 1989; Ferrara and Henzel, 1989; Connolly *et al.*, 1989b). Analysis of cDNA clones revealed heterogeneity in the VEGF coding region, and predicted the existence of 189-, 165-, and 121-residue forms of the mature human VEGF subunit, as well as 164- and 120-residue forms of the mature bovine VEGF subunit (Leung *et al.*, 1989; Keck *et al.*, 1989; Tischer *et al.*, 1989). Recently, a 164-residue form of the rat VEGF subunit has also been identified by amino acid sequencing and cDNA cloning (Conn *et al.*, 1990b). Taking into account the single site in VEGF for N-linked glycosylation, the 23-kDa subunit appears to correspond most closely to the size predicted for the 165 (164)-residue chain. Comparison of the amino acid sequences predicted from the cDNA clones with protein databases showed that VEGF is distantly related to both the A and B chains of PDGF, with complete conservation of the 8 cysteines involved in inter- and intra-chain disulfides in PDGF.

Here, we identify vascular smooth muscle (VSM) cells as an additional source of both VEGF mRNA and protein and

Many polypeptide mitogens such as basic fibroblast growth factor (bFGF)¹ and platelet-derived growth factor (PDGF)

* This work was supported in part by Small Business Innovation Research Grant HL44832-01 from the National Heart, Lung, and Blood Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M63971-M63978.

‡ Present address: Panlabs, Bothell, WA 98001.

¶ To whom correspondence should be addressed: California Biotechnology Inc., 2450 Bayshore Parkway, Mountain View, CA 94043. Tel.: 415-966-1550; Fax: 415-968-2438.

¹ The abbreviations used are: bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; VSM, vascular smooth muscle; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; bp, base pair(s); kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazinebis(ethanesulfonic acid).

characterize the multiple VEGF mRNA forms observed in VSM cells by polymerase chain reaction (PCR) and by cDNA cloning. Comparison of the cDNAs with the structure of the cloned human VEGF gene reported here shows that the VEGF

protein coding region is split among eight exons and that the three forms of the coding region arise by alternative exon splicing. The intron-exon pattern determined for the VEGF gene is somewhat related to that of the genes encoding the PDGF A and B chains.

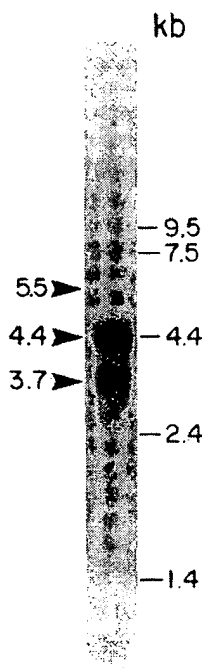


FIG. 1. Northern blot analysis of VEGF transcripts in fetal human VSM cells. Polyadenylated RNA (5 μ g) was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane. The membrane was probed with a 32 P-labeled 0.35-kb *EcoRI-HpaII* coding region fragment isolated from the bovine VEGF cDNA clone, λ ST800 (Tischer *et al.*, 1989). The hybridization was carried out in Rapid Hybridization Buffer (Amersham Corp.). The membrane was then washed at 52 $^{\circ}$ C in 0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS prior to autoradiography. VEGF mRNA species of 5.5, 4.4, and 3.7 kb were detected. Sizes of RNA markers (GIBCO-Bethesda Research Laboratories) are given in kilobases.

EXPERIMENTAL PROCEDURES

RNA Isolation and Northern Blot Analysis—Fetal human VSM cells (Gospodarowicz, 1990) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin sulfate; during growth of the cells, the medium was supplemented every other day with 1 ng/ml bFGF. U-937 cells obtained from the American Type Culture Collection (ATCC CRL 1593) were cultured in RPMI 1640 containing 10% fetal bovine serum, L-glutamine, and antibiotics as above to a density of 4×10^6 cells/ml. The U-937 cells were then diluted in growth medium to a density of 5×10^6 cells/ml and incubated with 20 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 3 days prior to RNA isolation.

To examine the effect of phorbol ester treatment on the level of VEGF transcripts in the fetal human VSM cells, 20 ng/ml of TPA were added to flasks of these cells at 3 days post-confluence (and 3 days past the last addition of bFGF). RNA was harvested from the cells either prior to TPA addition (control) or at various time intervals after TPA addition.

Total RNA was isolated from the cells by the guanidine thiocyanate method (Chirgwin *et al.*, 1979). Polyadenylated RNA was obtained by fractionation of the total RNA on oligo(dT)-cellulose (Type 3; Collaborative Research Inc.). Aliquots of total or polyadenylated RNA were subjected to denaturing electrophoresis on agarose-formaldehyde gels (Lehrach *et al.*, 1977) and transferred to either nitrocellulose filters (Schleicher & Schuell) or Hybond-N nylon membranes (Amersham Corp.), prior to hybridization to the nick-translated probes noted in the figure legends.

Protein Purification—Primary cultures of adult bovine VSM cells derived from the aorta were established as described previously (Gospodarowicz *et al.*, 1977, 1988). For large-scale culture of the cells in 530-cm² plates, seeding was at an initial cell density of 6×10^6 cells/plate in DMEM supplemented with 0.25 μ g/ml Fungizone, 50 μ g/ml gentamicin, 10% calf serum, and 1 ng/ml bFGF. Confluent monolayers were washed twice with DMEM alone prior to the addition of 150 ml/plate of DMEM supplemented with 2.5 μ g/ml Fungizone, 50 μ g/ml gentamicin, 5 μ g/ml insulin, 10 μ g/ml transferrin, and 0.1 ng/ml bFGF. After 48 h, culture fluids were collected and replaced with the same amount of fresh serum-free medium. Collections were made for two weeks without visible deterioration of the monolayer. VEGF

	70		80		90
	Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile				
PCR-1	<u>aaagctt</u> gag tgt gtc ccc act gag gag tcc aac atc acc atg cag ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA				
PCR-2	<u>aaagctt</u> gag tgt gtc ccc act gag gag tcc aac atc acc atg cag ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA				
PCR-3	<u>aaagctt</u> gag tgt gtc ccc act gag gag tcc aac atc acc atg cag ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA				
	100		110		
	Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val				
PCR-1	GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG AAG GAT AGA GCA AGA CAA GAA AAA TCA GTT				
PCR-2	GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG AAG GAT AGA GCA AGA CAA GAA AA				
PCR-3	GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG AAG GAT AGA GCA AGA CAA GAA AA				
	120		130		140
	Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser				
PCR-1	CGA GGA AAG GGA AAG GGG CAA AAA CCA AAG GGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT GCG TGT GCG CTT TGC TCA				
PCR-2	CGA GGA AAG GGA AAG GGG CAA AAA CCA AAG GGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT GCG TGT GCG CTT TGC TCA				
PCR-3	CGA GGA AAG GGA AAG GGG CAA AAA CCA AAG GGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT GCG TGT GCG CTT TGC TCA				
	150		160		170
	Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala				
PCR-1	GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CTT TGC AAG GCG				
PCR-2	GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CTT TGC AAG GCG				
PCR-3	GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CTT TGC AAG GCG				
	180		189		
	Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg				
PCR-1	AGG CAG CTT GAG TTA AAC GAA GGT ACT TGC AGA tgt gac aag ccg agg cgg tga gacggggcaggagcaagctt				
PCR-2	AGG CAG CTT GAG TTA AAC GAA GGT ACT TGC AGA tgt gac aag ccg agg cgg tga gacggggcaggagcaagctt				
PCR-3	AGG CAG CTT GAG TTA AAC GAA GGT ACT TGC AGA tgt gac aag ccg agg cgg tga gacggggcaggagcaagctt				

FIG. 2. DNA sequences of products obtained from PCR amplification of the variable (carboxyl-terminal) portion of the VEGF coding region in fetal human VSM polyadenylated RNA. The PCR products shown have the sequences expected for amplified copies of the carboxyl-terminal portion of the coding regions for VEGF₁₈₉ (PCR-1), VEGF₁₆₅ (PCR-2), and VEGF₁₂₁ (PCR-3). Primer sequences are shown in lower case letters, with the *HindIII* sites underlined. Dashes in the amplified sequences indicate nucleotides missing in PCR-2 and PCR-3 when compared with PCR-1. Predicted amino acids are numbered as in Keck *et al.* (1989) for the 189-residue form of VEGF (the PCR-2 nucleotide sequence encodes an asparagine at amino acid position 115, rather than lysine as in PCR-1 and PCR-3). The asterisk indicates an apparent polymorphism in the codon encoding amino acid 108.

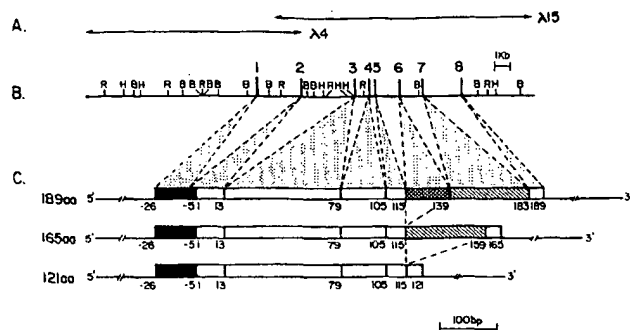


FIG. 3. Human VEGF gene structure. A, extents of the genomic DNA inserts contained in the clones λ 4 and λ 15. B, restriction map of the human VEGF gene and flanking regions, as established from clones λ 4 and λ 15. Sites are marked for the enzymes *EcoRI* (R), *HindIII* (H), and *BamHI* (B). The locations of exons 2-7 and of the coding region portions of exons 1 and 8 are indicated. C, the 189-, 165-, and 121-amino acid (aa) forms of VEGF arise by alternative splicing. Amino acids in each form are numbered below the schematic diagrams, starting with the mature amino terminus. The signal peptide is indicated by solid shading. Exon 6, which is only present in VEGF₁₈₉, is marked by cross-hatching; exon 7, present in VEGF₁₈₉ and VEGF₁₆₅, is indicated by diagonal lines.

protein was partially purified from the conditioned medium by a combination of heparin-Sepharose affinity chromatography and C_4 reversed-phase high-performance liquid chromatography (HPLC) as described (Gospodarowicz *et al.*, 1989). The proteins in the peak activity fractions from the HPLC column were electrophoresed on a reducing 12.5% acrylamide, sodium dodecyl sulfate (SDS) gel (Laemmli, 1970), transferred to a polyvinylidene difluoride membrane (Millipore), and sequenced on an Applied Biosystems 477A gas-phase protein sequencer with an on-line model 120A PTH Analyzer for amino acid identification.

PCR Analysis—By using the antisense primer 5' GCCAAGCTTGCTGCTCCTGCCCGGCTCACCAGCTCGGCTGTGTCACA 3' and a cDNA synthesis kit (Boehringer Mannheim), cDNA was synthesized from 5 μ g of fetal human VSM cell polyadenylated RNA. Following first strand cDNA synthesis, the reaction was extracted with phenol and chloroform and precipitated with ethanol. The cDNA was then amplified by 30 rounds of PCR (Saiki *et al.*, 1988) in a Perkin-Elmer Cetus DNA Thermal Cycler using the antisense primer above and a sense strand primer, 5' GCCAAGCTTGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAG 3'. Each primer contained a *HindIII* site near its 5' end, and the products of the PCR were digested with *HindIII* prior to fractionation on a 3% agarose gel. Fragments detected by ethidium bromide staining of the gel were eluted and cloned into the *HindIII* site in M13mp18 (Yanisch-Perron *et al.*, 1985) for sequence analysis by the dideoxynucleotide method (Sanger *et al.*, 1980).

Isolation of Genomic Clones—A human lung fibroblast genomic library in λ FIX (Stratagene) was screened on nitrocellulose filters by hybridization to the 789-base pair (bp) cDNA insert in clone λ ST800, which encodes a portion of bovine VEGF₁₂₀ (Tischer *et al.*, 1989). Hybridization was overnight at 37 °C in 40% formamide, 50 mM HEPES, pH 7.0, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.75 M NaCl, 75 mM sodium citrate, and 50 μ g/ml sheared salmon sperm DNA. The filters were washed at 50 °C in 0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS. An additional screening to search for the 5'-most coding region of VEGF was carried out with a ³²P-labeled 54-base oligonucleotide, 5' CTCTCTGGG-TACATTGGAGCCTTGCCCTTGCTGCTCTACCTTCACCATGCC AAG 3', designed based on the sequence of a bovine cDNA clone generated by primer extension across the 5' end of the bovine VEGF coding region. Hybridizations with the oligonucleotide and filter washes were as above, except that the hybridizations were carried out at room temperature in buffer lacking formamide. DNA from hybridizing phage was purified and subcloned into appropriate M13 cloning vectors for nucleotide sequencing (Sanger *et al.*, 1980) of both strands.

Primer Extension Analysis—Two synthetic antisense oligonucleotides, 5117 (5' CCTCTTTCTGCTGGTTTCCAAATCCACAG 3') and 5118 (5' CCTCGACTTCTCTCTGGAGCTCTTGTCTACC 3'), were labeled with [γ -³²P]ATP and polynucleotide kinase to 5×10^6

cpm/ μ g. In each primer extension reaction, approximately 5×10^5 cpm of one of the oligonucleotides was co-precipitated in ethanol with 1 μ g of U-937 cell polyadenylated RNA. In control reactions, the oligonucleotides were co-precipitated with 4 μ g of U-937 RNA which had been depleted of polyadenylated RNA by passage over oligo(dT)-cellulose. The samples were resuspended in 50 μ l of 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA, heated to 95 °C for 5 min, and annealed at 50 °C for 8 h. Following precipitation with ethanol, the samples were resuspended in 60 μ l of 33 mM KCl, 9 mM MgCl₂, 40 mM Tris-Cl, pH 8.5, and 0.7 mM each dATP, dCTP, dGTP, and dTTP. RNasin (60 units) and avian myeloblastosis virus reverse transcriptase (25 units; Boehringer Mannheim) were then added, and the reactions were incubated at 42 °C for 45 min. After extraction with phenol and chloroform and precipitation with ethanol, the products of each reaction were fractionated on a 6% polyacrylamide, 8 M urea gel alongside the products of a set of dideoxy sequencing reactions. The dideoxy sequence ladder was generated from a genomic subclone containing the 5' end of the VEGF gene, using as a sequencing primer the same oligonucleotide as was used for the primer extension.

RESULTS

Synthesis of VEGF in Cultured Vascular Smooth Muscle Cells—Since VSM cells are in close proximity to endothelial cells in blood vessels, we sought to determine whether they represent a potential source of VEGF activity that could act in a paracrine fashion on the vascular endothelium. Messenger RNA was isolated from fetal human VSM cells and found by Northern blot analysis (Fig. 1) to contain VEGF transcripts of 5.5, 4.4, and 3.7 kilobases (kb). This result is in contrast to the reports that bovine pituitary folliculo stellate cells contain only a 3.7-kb mRNA (Leung *et al.*, 1989) or 4.2-, 3.7-, and 3.4-kb mRNAs (Tischer *et al.*, 1989), whereas U-937 cells express an approximately 3.8-kb transcript (Keck *et al.*, 1989). VEGF transcripts were not detected in similar blots using polyadenylated RNA isolated from cultured capillary endothelial cells (data not shown).

To confirm that VSM cells are capable of producing VEGF protein as well as mRNA, conditioned medium was collected from bovine VSM cells, and the endothelial cell mitogen present in the medium was purified using heparin-Sepharose chromatography and C_4 reversed-phase HPLC, followed by electrophoresis on a 12.5% acrylamide, SDS gel. The proteins fractionated on the gel were transferred onto a polyvinylidene difluoride membrane, and the region of the membrane carrying a protein migrating at the same apparent molecular weight as bovine pituitary folliculo stellate cell VEGF (Gospodarowicz *et al.*, 1989) was excised for sequencing. The amino-terminal sequence of the protein on the membrane was determined to be Ala-Pro-Met-Ala-Glu-X-Gly-Gln-, in agreement with the amino-terminal sequence reported previously for bovine VEGF (Ala-Pro-Met-Ala-Glu-Gly-Gly-Gln-; Gospodarowicz *et al.*, 1989; Tischer *et al.*, 1989; Leung *et al.*, 1989).

Expression of Multiple Forms of the VEGF Coding Region in VSM Cells—A cDNA clone encoding the 189-amino acid form of human VEGF (VEGF₁₈₉) has been isolated from U-937 cells (Keck *et al.*, 1989), whereas cDNA clones encoding VEGF₁₈₉ and the 165- and 121-amino acid forms of human VEGF (VEGF₁₆₅ and VEGF₁₂₁) have been isolated from phorbol ester-activated HL60 cells (Leung *et al.*, 1989). Compared with VEGF₁₂₁, VEGF₁₆₅ has an extra 44 residues located six amino acids from the carboxyl terminus, and VEGF₁₈₉ has an additional 24 amino acids located immediately upstream of the 44-residue insert. To investigate the occurrence of this VEGF coding region heterogeneity in a cell type other than the promyelocytic leukemia HL60 cells, a bovine VEGF cDNA probe (Tischer *et al.*, 1989) was used to isolate multiple VEGF clones from a fetal human VSM library. Although many of these clones were found by nucleotide sequence analysis to be

- 100 AAGAGTAGCTCGCCGAGGGCCGAGGAGAGCGGCCCGCCACAGCCCGAGCCGGAGAGGGAGCGGAGCCGCGCCGCCCCCGGTGGGGCCCTCCGAAACC ATG AAC

-20 Exon1 -10
Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys
TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG gtaagcggtctgtgcct.....

-1 1 Exon2 10
..tctcttttctgtcctcag Trp Ser Gln Ala Ala Pro Met Ala Glu Gly Gly Gln Asn His His Glu V
TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA G gtagtccccctgtgctg

20 30
.....catcgctctctcatgcag al Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp
TG GTG AAG TTC ATG ATG GTC TAT CAG CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC

40 50 60
Ile Phe Gln Glu Tyr PCT Asp Glu Ile CAG Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys
ATC TTC CAG GAG TAC CAG ATG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CAG TGC GGG GGC TGC TGC TGC

70
Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln
AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC ATG CAG gtgggcatctttgggaa.....gcttcc

80 90 Exon4 100
ttcctttccag Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Glu Glu Met Arg Ser Phe Leu Gln His Asn Lys Cys Glu
ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA

110 Exon5 As
Cys Ar g Pro Lys Lys Asp Arg Ala Arg Gln Glu Ly
TGC AG gtgaggatgtagtcacg.....ctccctaccattgcag A CCA AAG AAA Gat AGA GCA AGA CAA GAA AA gtaagtggcctt

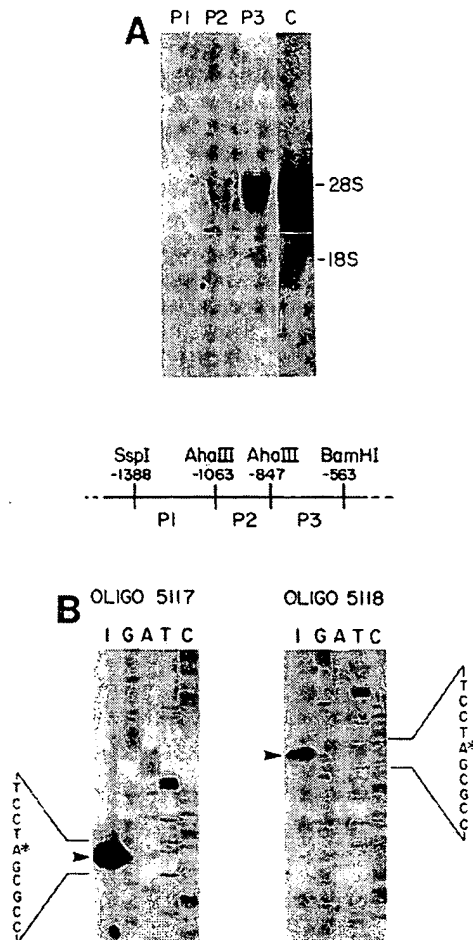
120 Exon6 130
gactt.....gtttttttattttccag s Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Ser Arg
A AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG

n 140
Tyr Lys Ser Trp Ser Va l Pro Cys Gly Pro Cys Ser Glu Arg Arg
TAT AAG TCC TGG AGC GT gtacgttggtgcccgt.....cttttgccctttttgcag T CCC TGT GGC CCT TGC TCA GAG CGG AGA

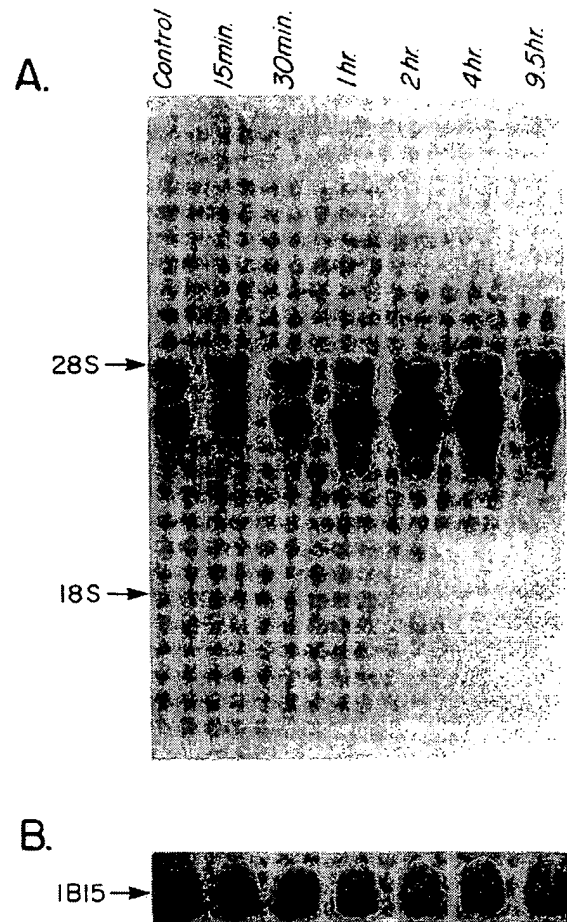
150 160 Exon7 170
Lys His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Cys Arg Lys Arg Gln Leu
AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA Asp TCG CGT TGC AAG CGC AGG CAG CTT

180 Exon8 189
Glu Leu Asn Glu Arg Thr Cys Ar s Cys Asp Lys Pro Arg Arg
GAG TTA AAC GAA CGT ACT TGC AG gtgtgttccccagagga.....ttttccattttccctcag A TGT GAC AAG CCG AGG CGG TGA

FIG. 4. Partial nucleotide sequence of the human VEGF gene. Nucleotides present in mature VEGF transcripts are shown in *upper case letters*; nucleotides present in the 5'-flanking region and introns are in *lower case*. Amino acids are numbered from the alanine residue corresponding to the amino terminus of the mature protein, and nucleotides are numbered from the translation start site. Regions closely matching transcriptional control consensus sequences are indicated as follows: *solid boxes*, Sp1 (5' GGGCGG 3' or 5' CCGCCC 3'); *open boxes*, TATA box (5' TATAAT 3').



copies of partially unspliced mRNAs, the sequence analysis showed that the spliced portions of the clones represented copies of three different sequences, corresponding to the coding regions for VEGF₁₈₉, VEGF₁₆₅, or VEGF₁₂₁ (data not shown).



The occurrence of the coding region heterogeneity in the fetal human VSM cells was confirmed by PCR analysis. In this experiment, oligonucleotides flanking the variable (carboxyl-terminal) portion of the coding region were used to prime a PCR synthesis from fetal human VSM single-stranded cDNA. The various products of the PCR reaction visible after agarose gel electrophoresis and ethidium bromide staining were excised from the gel, subcloned into M13mp18, and sequenced. This analysis revealed three products that had the sequences predicted for the PCR copies of the VEGF₁₈₉, VEGF₁₆₅, and VEGF₁₂₁ coding regions (Fig. 2).

Like HL60 cells, then, cultured fetal human VSM cells express three different forms of the VEGF coding region.

bold underlines, AP-1 (5' TGAC/GTCA 3'); **bold overlines**, AP-2 (5' CCCCAGGC 3'); **double underline**, nuclear factor I (5' GCCAAT 3'; Mitchell and Tjian, 1989); **double underline**, heat shock (5' CXXGAAXXTTCXNG 3'; Bienz and Pelham, 1986). The **dashed box** in the 5'-untranslated region marks the upstream ATG codon. The major site of transcription initiation as identified by primer extension analysis is indicated by a **bent arrow**.

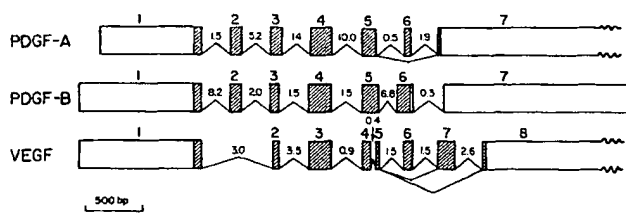


FIG. 7. Comparison of the intron-exon structure of the VEGF gene with those of the PDGF A and B chain genes. The number of each exon is given above each gene structure; coding regions are marked by diagonal lines and untranslated regions by open boxes. The precise lengths of the 3'-untranslated regions of PDGF A and VEGF are not known. Numbers between the exons refer to the size of each intron in kilobases. Both the normal and alternative exon splicing patterns are shown. The scale refers to the exon sequences only. Data for the PDGF A and B chains are from Rao *et al.* (1986) and Bonthron *et al.* (1988).

Similar PCR analyses using bovine folliculo stellate cell single-stranded cDNA as the template showed only the products corresponding to VEGF₁₆₄ and VEGF₁₂₀ (Tischer *et al.*, 1989), consistent with the fact that VEGF cDNA clones encoding a bovine equivalent to the 189-amino acid human form were not isolated from bovine folliculo stellate cDNA libraries (Leung *et al.*, 1989; Tischer *et al.*, 1989). This may be due to the different cell types analyzed, or it may indicate a species difference between human and bovine VEGF.

Isolation and Characterization of Human VEGF Genomic Clones—Two bacteriophage λ clones spanning portions of the human VEGF gene were isolated from a human lung fibroblast genomic library, using as probes either a bovine VEGF cDNA fragment or a synthetic oligonucleotide corresponding to the coding region for a portion of the secretion signal of bovine VEGF. The restriction enzyme maps for these two clones, λ 4 and λ 15, were determined for the enzymes *Hind*III, *Eco*RI, and *Bam*HI by a combination of single and double digests (Fig. 3). Restriction fragment lengths predicted from the maps were confirmed by Southern blot analysis of human genomic DNA (data not shown). The two genomic clones overlap (both contain exon 2) and define a contiguous stretch of approximately 28 kb. The coding domain for human VEGF is contained within approximately 14 kb.

The coding domains, intron-exon junctions, 5'-untranslated region, putative promoter region, and part of the 3'-untranslated region were sequenced (Fig. 4). A comparison of the sequence of the gene with that of the human cDNAs isolated previously (Keck *et al.*, 1989; Leung *et al.*, 1989), and characterized here from fetal human VSM cells, indicates that the coding sequence is interrupted by seven introns. The sequences of all intron-exon boundaries conform to the consensus splicing signals (Breathnach and Chambon, 1981). The locations of all of the exons except exons 6 and 8 on the genomic map shown in Fig. 3 were established directly from the nucleotide sequence analysis of subclones, in that the sequencing spanned restriction enzyme sites identified on the genomic map; exons 6 and 8 were located approximately through the use of exon-specific oligonucleotides to probe Southern blots of restriction enzyme-digested λ 15 DNA. The absence of additional introns in the 5'-untranslated region was confirmed by PCR (data not shown).

Comparison of the cDNA cloning and PCR results with the human VEGF gene sequence also indicated that the three forms of the VEGF coding region arise by alternative exon splicing (see Figs. 2 and 4). The alternative splicing involves exons 6 and 7 (Fig. 3); if neither exon is removed, VEGF₁₈₉ is generated; if only exon 6 is removed, VEGF₁₆₅ (with a lysine-to-asparagine change at residue 115) is generated; and if both

exons 6 and 7 are removed, VEGF₁₂₁ (with a lysine at residue 115) is generated. The 44-amino acid segment encoded by exon 7, which is present in both VEGF₁₈₉ and VEGF₁₆₅, contains 7 cysteines not found in the shortest VEGF form. This segment is also very basic in nature, containing 9 arginine and lysine residues. The 24-amino acid segment encoded by exon 6, present only in VEGF₁₈₉, is even more basic in nature, containing 12 lysine and arginine residues.

Analysis of the Human VEGF Gene Promoter—The 5' end of the human VEGF mRNA was mapped by Northern blot analysis as well as by primer extension using synthetic oligonucleotides. Northern blots of U-937 cell total RNA probed with a series of genomic restriction fragment probes (Fig. 5A) defined the approximate location of the transcriptional start site. The results indicated that probe P2 (nucleotides -1063 to -848 in Fig. 4) is the 5'-most probe which still hybridizes to the VEGF mRNA; probe P1 (nucleotides -1388 to -1064) gave no detectable signal on the Northern blot and apparently corresponds, therefore, to nontranscribed sequences. Primer extension analysis was then carried out with two synthetic antisense oligonucleotides whose 5' ends are 35 bases apart (nucleotides -749 to -720 and -714 to -685 in Fig. 4) to obtain a precise definition of the transcription start site. Results from this analysis (Fig. 5B) indicated that the human VEGF gene has a single major transcriptional start site, although several potential minor starts were also evident on longer exposures of the autoradiographs. By fractionating the primer extension products alongside matching DNA sequence ladders (Fig. 5B), the precise location of the major start site was found to be 1038 bp upstream from the ATG initiation codon.

VEGF Transcript Levels in VSM Cells Treated with TPA—The effect of phorbol ester treatment on the level of VEGF transcripts in fetal human VSM cells was assessed by exposing confluent cultures to 20 ng/ml TPA for various time periods. Polyadenylated RNA was then isolated, and the relative level of VEGF transcripts was determined by Northern blot analysis (Fig. 6). The results indicate that the amount of VEGF mRNA in the VSM cells rises in response to the TPA, reaching a maximum at 2-4 h.

DISCUSSION

Cultured VSM cells have been shown to synthesize VEGF mRNA and to secrete biologically active VEGF. This observation raises the possibility that VSM cells act as a source of VEGF that could function as a paracrine factor both to maintain the integrity of the vascular endothelium and to repair endothelial damage. Since VEGF binds heparin (although with a lower affinity than does bFGF; Gospodarowicz *et al.*, 1989; Ferrara and Henzel, 1989), locally synthesized VEGF might be stored in the extracellular matrix bound to heparin-like molecules, as appears to occur with bFGF (Vlodavsky *et al.*, 1987). Whether VSM cells make VEGF *in vivo* remains to be confirmed by *in situ* hybridization or by immunohistochemistry.

Structural characterization of the human VEGF gene shows that it comprises eight exons. Mature VEGF₁₈₉ retains coding sequence from exons 2-8 (exon 1 representing the majority of the secretion signal portion of the primary translation product), whereas VEGF₁₆₅ lacks the amino acids encoded by exon 6, and VEGF₁₂₁ lacks the residues encoded by exons 6 and 7. RNA species corresponding to all three forms have been detected in cultured VSM cells via cDNA cloning and PCR. VEGF protein isolated from various cell sources (Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989; Connolly *et al.*, 1989a, 1989b) has been reported to have a subunit molecular

mass of between 18 and 24 kDa, but individual proteins corresponding to the 189-, 165-, and 121-amino acid forms have not been definitively characterized. There is therefore as yet no direct evidence that the three proteins encoded by the three different VEGF coding region forms are actually synthesized *in vivo*.

The physical properties of the proteins that are predicted from the three coding sequences differ considerably. Both VEGF₁₈₉ and VEGF₁₆₅ have 16 cysteines; 8 of those are encoded by exons 3 and 4 (common to all VEGF forms), 7 are encoded by exon 7 (not present in VEGF₁₂₁), and 1 is encoded by exon 8 (common to all VEGF forms). There are also significant charge differences: 12 of the 24 codons in exon 6 (not present in VEGF₁₆₅ and VEGF₁₂₁) encode basic amino acids, and 9 of the 44 codons in exon 7 (not present in VEGF₁₂₁) encode basic amino acids. Thus, in addition to significant structural changes, isoelectric point variations should result from these differences, in that the shorter forms of VEGF are predicted to be more acidic. No biological activity differences have yet been reported reflecting these altered physical properties.

The locations of the intron-exon junctions in VEGF are compared in Fig. 7 to those in the PDGF A and B chains. Both PDGF A and B have very similar gene structures (Rao *et al.*, 1986; Bonthron *et al.*, 1988). An overall similarity in gene structure can also be observed between VEGF and the PDGFs, but significant differences do exist which are consistent with the low level (20–25%) of amino acid sequence relatedness between VEGF and the PDGFs. Exon 1 in both VEGF and the PDGFs contains a relatively long (approximately 840–1030 bp) 5'-untranslated region as well as the majority of the hydrophobic signal peptide domain. Following this, exon 2 in VEGF, and exons 2 and 3 in the PDGFs, represent a region with no sequence similarity. Exons 3 in VEGF and 4 in PDGF are comparable and contain 6 of the 8 conserved cysteine residues, but the precise sequences at the intron-exon boundaries are not conserved. VEGF exons 4 and 5 appear to correspond to exon 5 in the PDGFs; this region contains the two other conserved cysteines but the overall sequence relatedness is minimal. Exon 6 is a highly basic region in both PDGFs and in VEGF, and there is more sequence similarity in this region between VEGF and the PDGF A chain than between the two PDGFs (see Bonthron *et al.*, 1988; Betsholtz *et al.*, 1990).

In the case of PDGF A, but not PDGF B, an alternatively spliced form is found which lacks the residues encoded by exon 6. In this respect VEGF is, therefore, like PDGF A, since both VEGF₁₆₅ and VEGF₁₂₁ lack the exon 6 amino acids. The short form of PDGF A appears to be the exclusive PDGF A gene product in endothelial cells, and the long form is made by several transformed lines (Betsholtz *et al.*, 1986; Tong *et al.*, 1987; Collins *et al.*, 1987). Both the long and the short forms of PDGF A appear to be equally active, but it has been reported that the short form may be less efficiently secreted and dimerized (Beckman *et al.*, 1988; Collins *et al.*, 1987). In the case of VEGF, both the 189- and 165-residue forms have been expressed recombinantly and shown to be biologically active (Leung *et al.*, 1989; Keck *et al.*, 1989), indicating that, as with PDGF A, the exon 6-encoded amino acids are not essential for VEGF activity.

Exon 7 in VEGF, which encodes the domain with 7 cysteines, appears to have no counterpart in either PDGF chain. It will be interesting to determine whether the presence of this region confers VEGF₁₈₉ and VEGF₁₆₅ with biological properties in addition to mitogenicity, such as the ability to induce permeability in the vascular endothelium. Finally,

although there is no sequence similarity in this region, both PDGF A and VEGF have a short carboxyl-terminal coding region present in exons 7 and 8, respectively, followed by the 3'-untranslated region.

Similarly to both PDGF chains (Bonthron *et al.*, 1988; Rao *et al.*, 1986), the section of the 5'-untranslated region of VEGF immediately upstream of the translation initiation codon is very GC-rich. A GC-rich 5'-untranslated region has also been noted in other growth factor genes such as those encoding bFGF and transforming growth factor β (Abraham *et al.*, 1986; Derynck *et al.*, 1985) and may be involved in translational control. The 5'-untranslated region of VEGF contains a single ATG codon located 185–187 nucleotides upstream of the proposed initiating methionine codon. In this respect VEGF is again like the two PDGFs, both of which have three apparently nonutilized ATG codons in their 5'-untranslated regions. The significance of these ATGs is unknown but it has been proposed they might also have a role in translational control (Bonthron *et al.*, 1988).

Primer extension analysis mapped the single major transcriptional initiation site for the human VEGF gene to nucleotide -1038 (Fig. 4). Approximately 50 bp 5' to this site there is a cluster of four GC box sequences (5' GGGCGG 3' or 5' CCGCCC 3') at nucleotides -1096 to -1091, -1112 to -1107, -1123 to -1118, and -1133 to -1128. GC box sequences located close to transcription start sites in other genes have been shown to bind the transcription factor Sp1 (Gidoni *et al.*, 1984; Kadonaga *et al.*, 1986). Three of these four GC boxes near the transcription start site in the VEGF gene are also perfect matches to a consensus sequence for "strong" Sp1 binding sites (5' G/TGGGCGGG/AG/AC/T 3' or its complement 5' G/AC/TC/TCCGCCCC/A 3'; Kadonaga *et al.*, 1986). In addition, two other GC box sequences are found further upstream in the 5'-flanking region (nucleotides -1277 to -1272 and -2138 to -2133), and two more are present in the 5'-untranslated region (nucleotides -66 to -61 and -518 to -513). No TATA box-like promoter sequence (Corden *et al.*, 1980) is seen close to the start of transcription; the only match to this consensus sequence is found at nucleotides -2465 to -2461 in the 5'-flanking region, too far from the transcription start site to be of significance. The human VEGF gene, therefore, differs from the human PDGF genes which both have TATA box promoters (Rao *et al.*, 1986; Bonthron *et al.*, 1988). Also although the primer extension analysis carried out on the VEGF gene did reveal the possibility of several minor transcription start sites, it is unusual for a promoter with multiple closely spaced GC boxes as in the case of VEGF to have a single major transcription initiation site.

The sequence of the VEGF gene was scanned for additional consensus binding sites for other transcriptional control factors (reviewed in Mitchell and Tjian, 1989; Johnson and McKnight, 1989). The presence of four potential AP-1 binding sites (consensus 5' TGAC/GTCA 3') at nucleotides -620 to -614, -1528 to -1522, -2265 to -2258, and -2930 to -2924 (see Fig. 4) implied that the VEGF gene should be inducible by serum or by the phorbol ester TPA (Curran and Franza, 1988; Mitchell and Tjian, 1989). In addition, two potential AP-2 binding sites (consensus 5' CCCCAGGC 3' or 5' T/CCCCCA/CNG/CC/GG/C 3') at nucleotides -135 to -128 and -1875 to -1868 also indicated that VEGF gene expression should be inducible by TPA, as well as by cAMP (Imagawa *et al.*, 1987; Mitchell and Tjian, 1989). Consistent with these predictions, initial experiments with fetal human VSM cells indicate that TPA treatment indeed results in an increase in the level of VEGF mRNA (Fig. 6). The availability of cloned VEGF promoter sequences will allow a detailed examination

of this and other components involved in VEGF transcriptional regulation to take place.

Acknowledgments—We thank J. Iwasa for oligonucleotides, J. Miller for help with DNA sequencing, K. Wood for help with protein purification, K. Lau for protein sequencing, S. Pollitt and T. White for critical reading of the manuscript, E. Stoelting for artwork, and K. MacDonald for preparation of the manuscript.

REFERENCES

- Abraham, J. A., Whang, J. L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., and Fiddes, J. C. (1986) *EMBO J.* **5**, 2523–2528
- Beckman, M. P., Betsholtz, C., Heldin, C.-H., Westermarck, B., DiMarco, E., DiFiore, P. P., Robbins, K. C., and Aaronson, S. A. (1988) *Science* **241**, 1346–1349
- Betsholtz, C., Johnson, A., Heldin, C.-H., Westermarck, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J., and Scott, J. (1986) *Nature* **320**, 695–699
- Betsholtz, C., Rorsman, F., Westermarck, B., Ostman, A., and Heldin, C.-H. (1990) *Nature* **344**, 299
- Bienz, M., and Pelham, H. R. B. (1986) *Cell* **45**, 753–760
- Bonthron, D. T., Morton, C. C., Orkin, S. H., and Collins, T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1492–1496
- Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Collins, T., Bonthron, D. T., and Orkin, S. H. (1987) *Nature* **328**, 621–624
- Conn, G., Soderman, D. D., Schaeffer, M.-T., Wile, M., Hatcher, V. B., and Thomas, K. A. (1990a) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1323–1327
- Conn, G., Bayne, M. L., Soderman, D. D., Kwok, P. W., Sullivan, K. A., Palisi, T. M., Hope, D. A., and Thomas, K. A. (1990b) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2628–2632
- Connolly, D. T., Olander, J. V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B. L., Leimgruber, R., and Feder, J. (1989a) *J. Biol. Chem.* **264**, 20017–20024
- Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Siegel, N. R., Leimgruber, R. M., and Feder, J. (1989b) *J. Clin. Invest.* **84**, 1470–1478
- Corden, J., Wasyluk, B., Buchwaldet, A., Sassone-Corsi, P., Keding, C., and Chambon, P. (1980) *Science* **209**, 1406–1414
- Curran, T., and Franza, B. R., Jr. (1988) *Cell* **55**, 395–397
- Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B., and Goeddel, D. V. (1985) *Nature* **316**, 701–705
- Ferrara, N., and Henzel, W. J. (1989) *Biochem. Biophys. Res. Commun.* **161**, 851–858
- Gidoni, D., Dynan, W. S., and Tjian, R. (1984) *Nature* **312**, 409–413
- Gospodarowicz, D. (1990) *UCLA Symp. Mol. Cell. Biol.* **114**, 15–30
- Gospodarowicz, D., Moran, J., and Braun, D. (1977) *J. Cell. Physiol.* **91**, 377–385
- Gospodarowicz, D., Haaparanta, T., and Neufeld, G. (1988) *Eur. J. Cell Biol.* **46**, 144–151
- Gospodarowicz, D., Abraham, J. A., and Schilling, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 7311–7315
- Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* **51**, 251–260
- Johnson, P. F., and McKnight, S. L. (1989) *Annu. Rev. Biochem.* **58**, 799–839
- Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci.* **11**, 20–23
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989) *Science* **246**, 1309–1312
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16**, 4743–4751
- Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) *Science* **246**, 1306–1309
- Levy, A. P., Tamargo, R., Brem, H., and Nathans, D. (1989) *Growth Factors* **2**, 9–19
- Mitchell, P. J., and Tjian, R. (1989) *Science* **245**, 371–378
- Plouët, J., Schilling, J., and Gospodarowicz, D. (1989) *EMBO J.* **8**, 3801–3806
- Rao, C. D., Igarashi, H., Chiu, I.-M., Robbins, K. C., and Aaronson, S. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 2392–2396
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239**, 487–491
- Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., and Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178
- Senger, D. R., Gall, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) *Science* **219**, 983–985
- Senger, D. R., Perruzzi, C. A., Feder, J., and Dvorak, H. F. (1986) *Cancer Res.* **46**, 5629–5632
- Sternfeld, M. D., Hendrickson, J. E., Keeble, W. W., Rosenbaum, J. T., Robertson, J. E., Pittelkow, M. R., and Shipley, G. D. (1988) *J. Cell. Physiol.* **136**, 297–304
- Tischer, E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J. C., and Abraham, J. A. (1989) *Biochem. Biophys. Res. Commun.* **165**, 1198–1206
- Tong, B. D., Auer, D. E., Jaye, M., Kaplow, J. M., Ricca, G., McConathy, E., Drohan, W., and Deuel, T. F. (1987) *Nature* **328**, 619–621
- Vlodavsky, I., Folkman, J., Sullivan, R., Friedman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2292–2296
- Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene (Amst.)* **33**, 103–119

cerebral malaria, and indicate that sequestration is commonly but not invariably associated with detectable intra- and perivascular pathology. Further studies may elucidate whether different pathogenic mechanisms accompany the various stages of parasite development in sequestration sites, and may facilitate the design of new strategies for the prevention and treatment of malaria.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by the US National Institutes of Health (RO1 AO34969) and The Wellcome Trust (042390/Z/94). W.F. is partly supported by the US National Cancer Institute/National Institutes of Health (CA 90301). Many clinicians provided patient care (see Supplementary Note online). For conducting the autopsies, we thank mortuary attendants D. Kotokwa and W. Namanya, and pathologists M. Appleton, D. Barrowcliff, R. Bazinet, D. Connor, T. Kovan, F. MacInnes, H. Manz, D. Milner, A. Oviedo, U. Mahadeva, E. Pollina, C. Richards, C. Richert, L. Stein, R. Tozer, G. Vischer and V. White. We thank

the patients' families for the privilege of conducting these autopsies, A. Indurkha for early statistical input, S. Rogerson for reviewing the manuscript and P. Marsh for nutritional support.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 10 November; accepted 31 December 2003

Published online at <http://www.nature.com/naturemedicine/>

1. Newton, C.R.J.C., Taylor, T.E. & Whitten, R.O. *Am. J. Trop. Med. Hyg.* **58**, 673–683 (1998).
2. Marsh, K., English, M. & Crawley, J. *Ann. Trop. Med. Parasitol.* **90**, 395–402 (1996).
3. Silamut, K. & White, N.J. *Trans. R. Soc. Trop. Med. Hyg.* **87**, 436–443 (1993).
4. Bolton, C.F., Young, G.B. & Zochodne, D.E. *Ann. Neurol.* **33**, 94–100 (1993).
5. Breiman, L., Friedman, J.H., Olshen, R.A. & Stone, C.J. in *Classification and Regression Trees* (Chapman and Hall/CRC, London, 1993).
6. Lewallen S. *et al. Trans. R. Soc. Trop. Med. Hyg.* **93**, 619–622 (1999).

Direct evidence that the VEGF-specific antibody bevacizumab has antivasular effects in human rectal cancer

Christopher G Willett¹, Yves Boucher^{1,10}, Emmanuelle di Tomaso^{1,10}, Dan G Duda^{1,10}, Lance L Munn^{1,10}, Ricky T Tong^{1,10}, Daniel C Chung², Dushyant V Sahani³, Sanjeeva P Kalva³, Sergey V Kozin⁴, Mari Mino⁴, Kenneth S Cohen⁵, David T Scadden⁵, Alan C Hartford¹, Alan J Fischman⁶, Jeffrey W Clark⁷, David P Ryan⁷, Andrew X Zhu⁷, Lawrence S Blazskowsky⁷, Helen X Chen⁹, Paul C Shellito⁸, Gregory Y Lauwers⁴ & Rakesh K Jain¹

The effects of vascular endothelial growth factor (VEGF) blockade on the vascular biology of human tumors are not known. Here we show here that a single infusion of the VEGF-specific antibody bevacizumab decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of viable, circulating endothelial and progenitor cells, and increases the fraction of vessels with pericyte coverage in rectal carcinoma patients. These data indicate that VEGF blockade has a direct and rapid antivasular effect in human tumors.

VEGF has a crucial role in physiological and pathological angiogenesis^{1–3}. Although VEGF blockade, alone or in combination with cytotoxic therapies, is being tested in a number of clinical trials⁴, including the first successful phase 3 clinical trial⁵, the effects of anti-VEGF treatment on the vascular biology of human tumors are not known. To this end, we recently initiated a National Cancer Institute-sponsored phase 1 clinical trial that integrates the VEGF-specific antibody bevacizumab (Avastin; Genentech) into a contemporary treatment program of pre-

operative chemotherapy and radiation therapy followed by surgery, for patients with primary and nonmetastatic rectal cancer. To gain insight into the mechanisms of action of bevacizumab, we designed the trial to evaluate the effects of bevacizumab alone on (i) tumor physiology (blood perfusion, blood volume, permeability–surface area product, microvascular density (MVD), perivascular coverage, interstitial fluid pressure (IFP) and 18-fluorodeoxyglucose (FDG) uptake); (ii) systemic response (VEGF level in blood, number of circulating endothelial cells (CECs) and progenitor cells); and (iii) tumor response (see Supplementary Note online for methods).

Six patients with primary and locally advanced adenocarcinoma of the rectum have been enrolled in a preoperative treatment protocol of bevacizumab administration alone (5 mg/kg intravenously), followed after 2 weeks—the approximate half-life of bevacizumab in circulation—by concurrent administration of bevacizumab with 5-fluorouracil and external beam radiation therapy to the pelvis and surgery, 7 weeks after treatment completion. Twelve days after bevacizumab infusion, flexible sigmoidoscopy (Fig. 1) revealed that bevacizumab induced tumor regression of >30% in patient 1, and no change in tumor size in the remaining five patients. Functional computed tomography (CT) scans at this time point indicated significant decreases in tumor blood perfusion (40–44%; $P < 0.05$) and blood volume (16–39% in four of five patients analyzed; $P < 0.05$; Figs. 1b and 2; see Supplementary Table 1 online for group statistics). This was accompanied by a significant decrease in tumor MVD (29–59% in five patients analyzed; $P < 0.05$; Fig. 2d). These three sets of data provide direct evidence of the antivasular effects of bevacizumab in human tumors, which is in line with preclinical findings^{6,7}.

Twelve days after bevacizumab treatment, IFP was reduced in four of four patients (Fig. 2f) and overall mean IFP decreased significantly from 15.0 ± 2.0 to 4.0 ± 2.2 mm Hg ($P < 0.01$). The decrease in IFP is in concert with our preclinical findings⁶. Elevated IFP, a hallmark of solid tumors, is a result of abnormalities in tumor vessels (such as abnormal structure of the vessel wall). The decrease in IFP after anti-VEGF treatment may be a result of 'normalization'; that is, the resumption of nor-

¹Department of Radiation Oncology, ²Division of Gastroenterology, ³Department of Radiology, ⁴Department of Pathology, ⁵AIDS Research Center/Experimental Hematology, ⁶Department of Nuclear Medicine, ⁷Division of Hematology/Oncology and ⁸Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, USA. ⁹Cancer Therapy Evaluation Program, National Cancer Institute, Bethesda, Maryland 20852, USA. ¹⁰These authors contributed equally to this work. Correspondence should be addressed to C.G.W. (cwillett@partners.org) or R.K.J. (jain@steels.mgh.harvard.edu).

Published online 25 January 2004; doi:10.1038/nm988



BRIEF COMMUNICATIONS

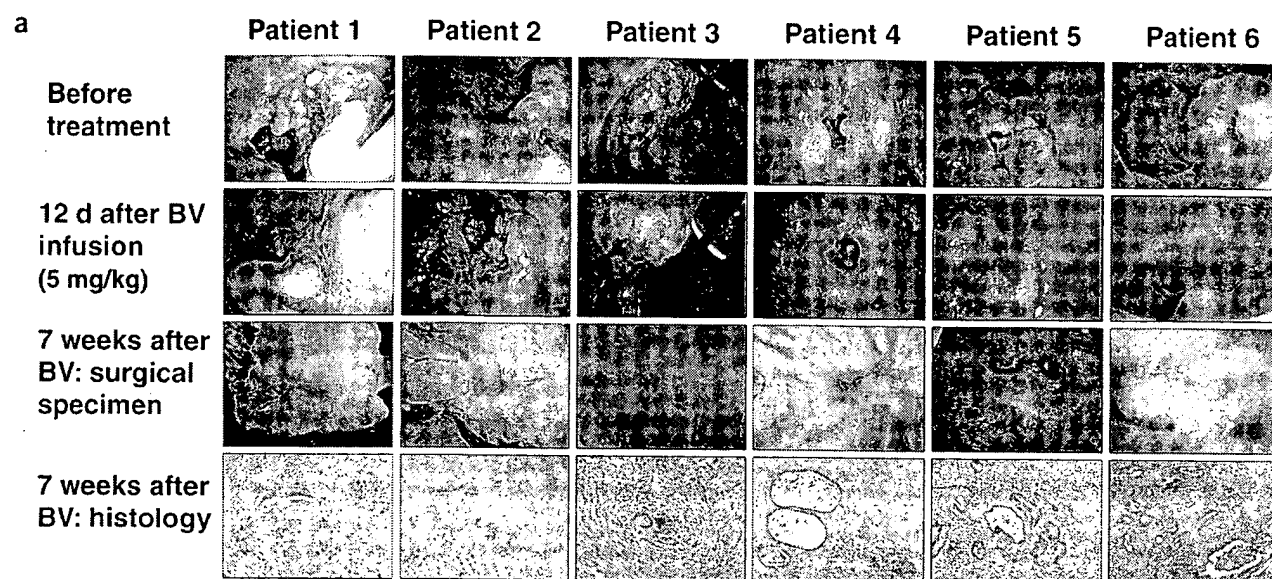
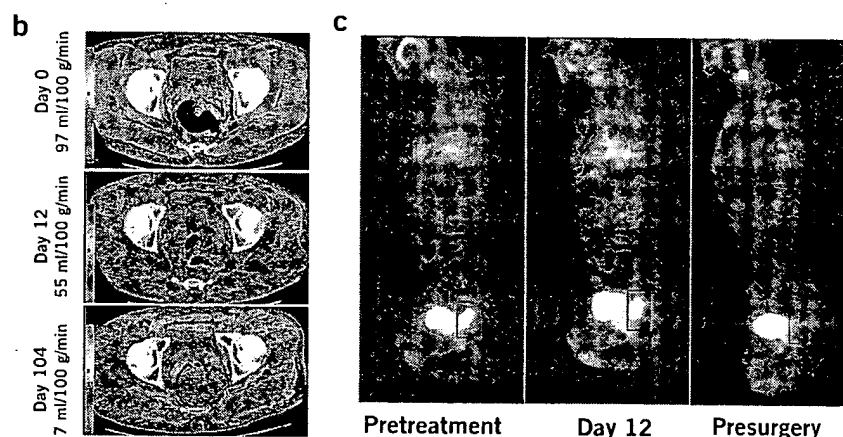


Figure 1 Effect of treatment on tumors in patients who completed entire combined treatment regimen, and surgery. (a) Endoscopic and pathological evaluation of rectal tumors. Surgical specimens showed grade II tumor regression in patients 1–5 and grade III in patient 6, by Mandard criteria (see **Supplementary Note**). Endoscopic image (instead of surgical specimen) was taken for patient 6, 3.5 weeks before surgery. BV, bevacizumab. (b) Representative functional CT images of blood perfusion before treatment (day 0), after bevacizumab (day 12) and after completion of treatment (day 104) in patient 5. (c) Tumor FDG uptake before treatment (pretreatment), 12 d after bevacizumab treatment and 6–7 weeks after completion of all neoadjuvant therapy (presurgery). Sagittal projections of FDG-PET scans for patient 1 are shown. Tumor is outlined in box, posterior to bladder.



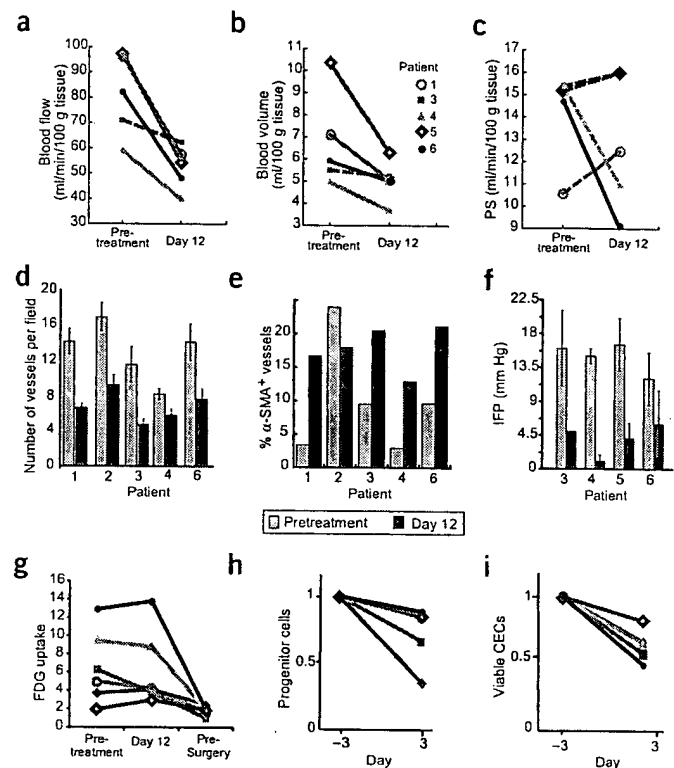
mal function in the tumor vasculature⁸. The increased fraction of vessels positive for α -smooth muscle actin in four of five patients (Fig. 2e and **Supplementary Fig. 1** online) is supportive of vascular normalization⁹. As a result of the decrease in vascular volume and MVD, one would expect a reduction in vascular surface area, and hence a lowering of the permeability–surface area product. Surprisingly, four of five patients had no significant changes in permeability–surface area product (Fig. 2c and **Supplementary Table 1**), providing indirect evidence for improved extravasation of the CT contrast agent from the normalized vasculature. Finally, on day 12 the follow-up positron emission tomography (PET) scans indicated no change in tumor FDG uptake in five patients, and showed a 40% decrease in patient 3 (Fig. 2g). Collectively, these data suggest that the efficiency of blood vessels after bevacizumab treatment is improved. These clinical findings are consistent with previous preclinical data on tumor oxygenation⁶ and drug uptake¹⁰ after anti-VEGF treatment.

In addition to being a mitogen and survival factor for endothelial cells, VEGF mobilizes progenitor cells from the bone marrow into the circulation¹¹. VEGF blockade decreased the number of progenitor cells circulating on day 3 (Fig. 2h). No decrease in circulating VEGF

(plasma, serum and urine) was detected with the current assay (see **Methods**; data not shown). After bevacizumab treatment, the number of CD31^{bright}CD45⁺ cells, but not the total number of CD31⁺CD45⁺ cells, decreased in all patients (Fig. 2h). Further analysis indicated that CD31^{bright}CD45⁺ cells represented viable CECs (**Supplementary Fig. 2** online). An increase in the number and viability of CECs was recently found in lymphoma and breast cancer patients¹². In addition, preclinical studies indicate that CEC kinetics might serve as a surrogate marker of response to treatment¹³. Based on these data, we suggest that the kinetics of progenitor cells or viable CECs in peripheral blood should be explored as an early indicator of tumor response to anti-VEGF agents.

Six weeks after completion of the bevacizumab, radiation therapy and chemotherapy regimen, follow-up PET scans (Fig. 1c) showed decreased tumor FDG uptake compared with pretreatment values in five patients (Fig. 2g). Tumor FDG uptake in patient 5 was low, and similar before and at the end of therapy (Fig. 2g). Notably, all six patients completed the combined treatment without dose-limiting toxicity, and underwent surgery without perioperative or postoperative complications. Macroscopic and histologic analysis of the sur-

Figure 2 Effect of a single injection of bevacizumab on tumor vasculature and FDG uptake. Parameters were obtained pretreatment and after one bevacizumab infusion. (a–c) Blood perfusion (a), blood volume (b) and permeability–surface area product (PS; c). Significant decreases after treatment are indicated by solid lines ($P < 0.05$ by t -test). Blood flow and blood volume decreased significantly in four of the patients. (d) Microvascular density. All patients showed significant decreases after treatment ($P < 0.05$ by t -test). (e) Fraction of vessels with pericyte coverage. The difference in the fraction of vessels positive for α -smooth muscle actin (α -SMA) in patient 2 was identified as an outlier by the Extreme Studentized Deviate test. Paired t -test analyses of the mean values that included and excluded the data of patient 2 had $P < 0.09$ and 0.001 , respectively. (f) Mean tumor IFP decreased significantly after bevacizumab ($P < 0.01$ by paired t -test). (g) Tumor FDG uptake before treatment, on day 12 and presurgery (day 93), normalized for muscle values. On day 12 after bevacizumab treatment, a 40% decrease was observed in patient 3, and no change in the other patients. Lower levels were found in all patients before surgery except for patient 5, who had low levels throughout the treatment. In comparison to pretreatment and day 12 values, the median standard uptake value was significantly lower on day 93 ($P < 0.01$; Supplementary Table 1). (h) Circulating progenitor/stem cells (AC133⁺; left) and viable CECs (right) in peripheral blood. Samples were run to acquire 50,000 events in the mononuclear/lymphocyte gate. For both cell populations, bevacizumab induced a significant decrease in mean values ($P < 0.05$ by Wilcoxon signed-rank test). Key in b applies to a, c, g–i.



gical specimens revealed a marked response in all six patients, with only microscopic disease in five of the patients (Fig. 1a and Supplementary Note).

High doses of bevacizumab are more effective when used as monotherapy for highly VEGF-dependent tumors such as renal-cell carcinoma¹⁴. Our data from six consecutive patients show for the first time that even at low doses, bevacizumab alone can decrease perfusion, MVD and IFP in a solid tumor, and decrease the number of CECs. The decrease in IFP and increase in the fraction of vessels with pericyte coverage support the normalization hypothesis⁸. This normalization process may retard the shedding of metastatic cells in the circulation and improve the delivery of therapeutic agents in tumors. Bevacizumab may also sensitize the endothelium to cytotoxic agents. Collectively, these mechanisms may explain the unprecedented efficacy of bevacizumab in recent clinical trials, as well as the possible synergistic or additive interaction between antiangiogenic and cytotoxic therapies that has been observed in preclinical settings for more than a decade^{4,15}. The identification of valid surrogate markers for antiangiogenic therapy, alone or combined with cytotoxic therapies, has been elusive. The results of this phase 1 study will hopefully facilitate and stimulate future research in this area.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This study was supported by two National Cancer Institute grants (R21 CA099237 to C.G.W. and PO1 CA80124 to R.K.J.). D.G.D. is a Cancer Research Institute fellow. R.T.T. is a fellow of the Susan G. Komen Breast Cancer Foundation. We thank T. Lee for his contribution to the CT analysis, M. Ancukiewicz, T.P. Padera and W. Strauss for helpful comments, and J. Toorodman for the ELISAs.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 18 November 2003; accepted 5 January 2004

Published online at <http://www.nature.com/naturemedicine/>

1. Carmeliet, P. & Jain, R.K. *Nature* **407**, 249–257 (2000).
2. Dvorak, H.F. *J. Clin. Oncol.* **20**, 4368–4380 (2002).
3. Ferrara, N., Gerber, H.P. & LeCouter, J. *Nat. Med.* **9**, 669–676 (2003).
4. Kerbel, R. & Folkman, J. *Nat. Rev. Cancer* **2**, 727–739 (2002).
5. McCarthy, M. *Lancet* **361**, 1959 (2003).
6. Lee, C. *et al. Cancer Res.* **60**, 5565–5570 (2000).
7. Yuan, F. *et al. Proc. Natl. Acad. Sci. USA* **93**, 14765–14770 (1996).
8. Jain, R.K. *Nat. Med.* **7**, 987–989 (2001).
9. Jain, R.K. *Nat. Med.* **9**, 685–693 (2003).
10. Wildiers, H. *et al. Br. J. Cancer* **88**, 1979–1986 (2003).
11. Rafii, S. *et al. Nat. Rev. Cancer* **2**, 826–835 (2002).
12. Mancuso, P. *et al. Blood* **97**, 3658–3661 (2001).
13. Monestiroli, S. *et al. Cancer Res.* **61**, 4341–4344 (2001).
14. Yang, J.C. *et al. N. Engl. J. Med.* **349**, 427–434 (2003).
15. Teicher, B.A. *Cancer Metastasis Rev.* **15**, 247–272 (1996).



Identification of a *c-fos*-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family

(Fos/cell transformation)

MAURIZIO ORLANDINI, LUCIA MARCONCINI, REBECCA FERRUZZI, AND SALVATORE OLIVIERO*

Dipartimento di Biologia Molecolare Università di Siena, Centro Ricerche IRIS, via Fiorentina 1, 53100 Siena, Italy

Communicated by William J. Rutter, University of California, San Francisco, CA, July 24, 1996 (received for review June 22, 1996)

ABSTRACT Using a mRNA differential screening of fibroblasts differing for the expression of *c-fos* we isolated a *c-fos*-induced growth factor (FIGF). The deduced protein sequence predicts that the cDNA codes for a new member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. Northern blot analysis shows that FIGF expression is strongly reduced in *c-fos*-deficient cells. Transfection of exogenous *c-fos* driven by a constitutive promoter restores the FIGF expression in these cells. In contrast, both PDGF and VEGF expression is unaffected by *c-fos*. FIGF is a secreted dimeric protein able to stimulate mitogenic activity in fibroblasts. FIGF overexpression induces morphological alterations in fibroblasts. The cells acquire a spindle-shaped morphology, become more refractive, disorganized, and detach from the plate. These results imply that FIGF is a downstream growth and morphogenic effector of *c-fos*. These results also suggest that the expression of FIGF in response to *c-fos* activation induces specific differentiation patterns and its aberrant activation contributes to the malignant phenotype of tumors.

The *c-fos* protooncogene plays a central role in the nuclear response to stimulatory signals that regulate cellular proliferation and differentiation. It codes for a nuclear protein that belongs to the AP-1 family of transcription factors. AP-1 factors are part of the bZip family of transcription factors which can form homo- and heterodimers and activate transcription by binding the DNA at AP-1 sites (1, 2). AP-1 is composed of dimeric complexes formed between Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) proteins which are induced by many cellular stimuli including growth factors, cytokines, T-cell activators, and UV irradiation (3). As a member of the immediate-early genes, *c-fos* expression is rapidly and transiently increased in response to extracellular signals. The role of *c-fos* during development has been studied by the generation of *c-fos*-deficient mice (4, 5). *c-fos* knockout mice are viable but show a range of tissue specific developmental defects including osteopetrosis, delayed gametogenesis, and lymphopenia.

Continuous expression of *c-fos* causes transformation of fibroblasts and loss of polarity of epithelial cells *in vitro* (6), and induces the formation of chondroblastic osteosarcomas when it is expressed under the control of ubiquitous promoters in transgenic mice (ref. 7 and references therein). Tumors obtained from *c-fos*-deficient cells fail to undergo malignant progression even if they are carrying the activated v-H-ras (8). These experiments suggest an essential role of *c-fos* in the malignant tumor development. *c-fos* contribution to differentiation and tumor progression is most probably due to the activation of specific target genes. These may play a role in

differentiation, in cell transformation, and/or malignant progression of tumors. A large number of genes have been shown to contain functional AP-1 sites in their regulatory regions. These include *c-jun* (2), the adipocyte P2 gene (9), type I collagenase (10), and stromelysin (11). Different strategies have been adopted to identify new *c-fos* target genes. The generation of a hormonally regulated c-Fos-estrogen receptor chimera allowed the isolation of the *c-fos* responsive gene Fit-1 (12) which codes for a membrane-associated protein. Reversion of the v-fos-dependent transformed phenotype in rat cells allowed the isolation of Fte-1 (13), a protein probably involved in protein import into mitochondria.

To isolate new *c-fos*-responsive genes we utilized cells differing only for the expression of *c-fos*. By mRNA differential display we compared the expression pattern of *c-fos*-deficient fibroblasts with cells derived from their wild-type siblings. In this report we describe the isolation of a cDNA that is strongly induced by *c-fos*. The cDNA sequence shows that it codes for a putative growth factor related to the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. The protein, which was named FIGF (for *c-fos*-induced growth factor), is secreted and shows autocrine mitogenic and morphogenic effects on fibroblasts.

MATERIALS AND METHODS

Cells and Cell Culture. *c-fos* (–/–)-deficient fibroblasts, obtained from *c-fos* knockout mice (5, 14) and *c-fos* (+/+) fibroblasts derived from their wild-type siblings were grown in DMEM supplemented with 10% fetal calf serum (FCS). The *c-fos* (–/–) cells, which express constitutively *c-fos*, were cultured in DMEM supplemented with 10% FCS and G418 (Geneticin; GIBCO/BRL) at 400 µg/ml. Stable clones constitutively expressing FIGF were obtained by cotransfection of an FIGF expression vector together with a plasmid containing the hygromycin resistance gene under the control of the simia virus 40 promoter (SO166). Transfectants were selected in DMEM supplemented with 10% FCS and hygromycin B (Calbiochem) at 300 µg/ml. The FIGF expression vector was constructed by the cloning of the FIGF cDNA under the control of the cytomegalovirus (CMV) promoter in the plasmid pcDNAIII-Δ neo (kindly provided by L. D'Adamio, National Institutes of Health).

Differential Display and Cloning of FIGF cDNA. *c-fos* (–/–) and *c-fos* (+/+) cells were maintained in DMEM containing 0.5% FCS for 48 h and then subjected to serum treatment. After 2 h of 10% serum induction, total cellular

Abbreviations: PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; FCS, fetal calf serum; CMV, cytomegalovirus; MEF, mouse embryo fibroblast.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U99572).

*To whom reprint requests should be addressed. e-mail: oliviero@iris02.biocine.it.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

RNA was extracted by the guanidinium thiocyanate method (15) and subjected to the differential display technique (16–18). The amplified cDNA fragments were compared in non-denaturing gels (19). The differentially expressed cDNAs were reamplified, cloned into pGEM-T vector (Promega), and used as probe in Northern blot assay. A fibroblast cDNA library was generated by oligo-dT reverse transcription of poly(A)⁺ RNA from a cell clone constitutively expressing *c-fos*, and cloned into Uni-Zap vector (Stratagene). A partial cDNA fragment (273 bp), whose corresponding mRNA was induced by *c-fos*, was labeled with [³²P]dCTP by random prime labeling and used to screen the library. The longest cDNA isolated was sequenced on both strands by the dideoxy DNA sequencing method (United States Biochemical).

Northern Blot Analysis. Total RNA (10 µg) was run on denaturing formaldehyde-agarose gel and transferred to nylon membranes. Filters were hybridized with [³²P]-labeled probes at 60°C in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA. The filters were washed for two 30-min periods at 60°C in 40 mM sodium phosphate (pH 7.2), 1% SDS, and 1 mM EDTA and exposed to x-ray film or analyzed by using a PhosphorImager (Molecular Dynamics).

Production of Bacterial FIGF and Anti-FIGF Antibodies. The FIGF protein was expressed in *Escherichia coli* under the control of the T5 promoter. The cDNA fragment, from the coding region of FIGF, was generated by PCR from the

methionine residue at position +40 and cloned into the pQE-31 vector (Qiagen, Chatsworth, CA) to obtain a fusion protein with a N-terminal histidine tag. The protein was expressed in TG1 bacteria (pREP+) by induction for 4 h at 37°C in the presence of 2 mM isopropyl β-D-thiogalactopyranoside. The recombinant protein was exclusively localized in inclusion bodies and was purified on a column of Ni-NTA-resin under denaturing conditions, according to the manufacturer's protocols (Qiagen). To produce partially refolded FIGF protein, the purified recombinant protein was treated as described (20, 21). Briefly the protein concentration was adjusted to 0.4 mg/ml and completely reduced in the presence of 8 M urea, 2% 2-mercaptoethanol for 1 h at 37°C. The reduced protein was dialyzed against a solution containing 50 mM Tris-HCl (pH 8.0), 1 M urea, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione for 2 days, and against a solution containing 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl for 1 day. Polyclonal antibodies were raised by injecting New Zealand White rabbits with 200 µg of recombinant FIGF in form of denatured protein in complete Freund's adjuvant. Antiserum was prepared after four injections in incomplete Freund's adjuvant at 3-week intervals.

Expression of FIGF in COS-7 Cells. COS-7 cells were transfected with an expression vector (pcDNAIII; Invitrogen) containing the FIGF coding sequence by using calcium phosphate precipitation. Cells were metabolically labeled with

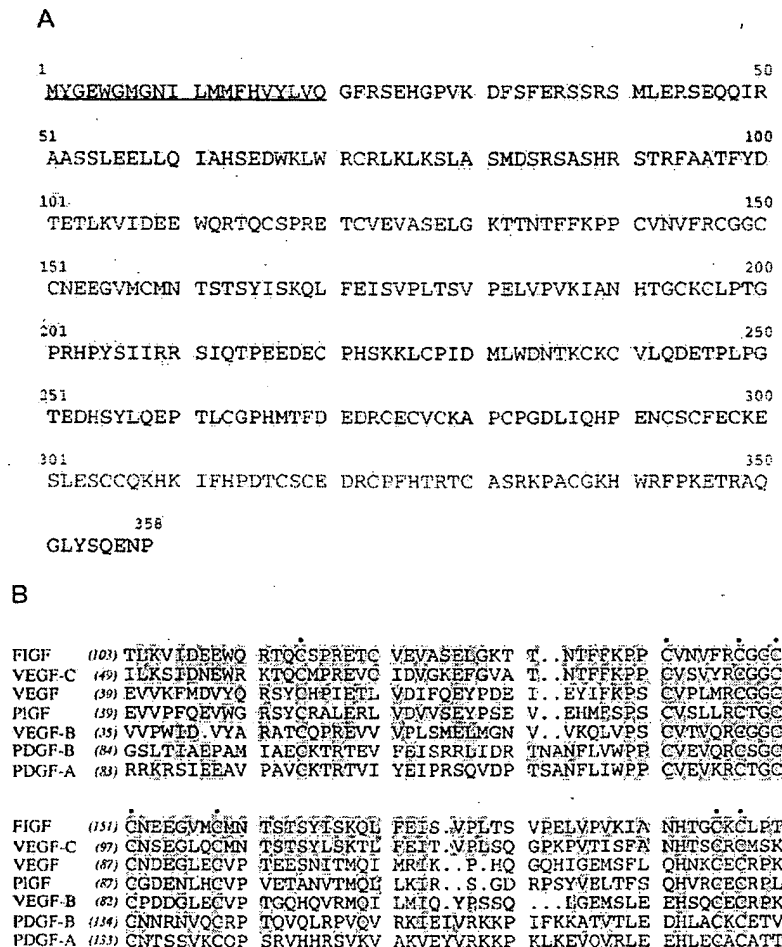


FIG. 1. (A) Deduced amino acid sequence of mouse FIGF. The putative secretory signal peptide rich in hydrophobic residues is underlined. (B) Alignment of the FIGF protein with the conserved domain of the PDGF/VEGF family of growth factors. Amino acid residues identical to FIGF are boxed. Dots indicate the cysteine residues which are characteristic of these growth factors (22). Numbers on the left indicate amino acid positions relative to the initiator methionine residue of each protein.

[35 S]methionine and [35 S]cysteine (Amersham) at 100 mCi/ml (1 Ci = 37 GBq) for 1 h and chased with cold methionine and cysteine. After the chase period medium was collected and cells were lysed in 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, and 4 μ g/ml phenylmethylsulfonyl fluoride. Conditioned media and cell lysates were immunoprecipitated separately with anti-FIGF polyclonal antibodies. Immune complexes were collected on protein A-Sepharose beads (Pharmacia) and separated by 12% SDS/PAGE in the presence of 2% 2-mercaptoethanol.

Mitogenic Assay for FIGF. Conditioned medium containing FIGF was collected from cells transfected with the appropriate expression vectors or with vector alone. *c-fos* ($-/-$) cells were plated into 96-well plates at the density of 5×10^3 cells/well in DMEM supplemented with 0.5% FCS and incubated for 48 h. Mouse embryo fibroblasts (MEFs) were obtained from 13- to 15-day embryos of B6D2F1 mice. The embryos were sacrificed, rinsed in Hanks' balanced salt solution and trypsinized for 30 min at 37°C. MEFs were grown in DMEM supplemented with 10% FCS. Second-passage MEFs were plated into 96-well plates at the density of 7×10^3 cells/well in DMEM containing 0.5% FCS and incubated for 30 h. Conditioned media or purified proteins were added to the wells and cells were stimulated for 14 h. [3 H]Thymidine (2.5 μ Ci/ml) diluted in DMEM without serum was added to the cells for a period of 8 h. Cells were washed with PBS, trypsinized, and the incorporated radioactivity was determined by liquid scintillation counting.

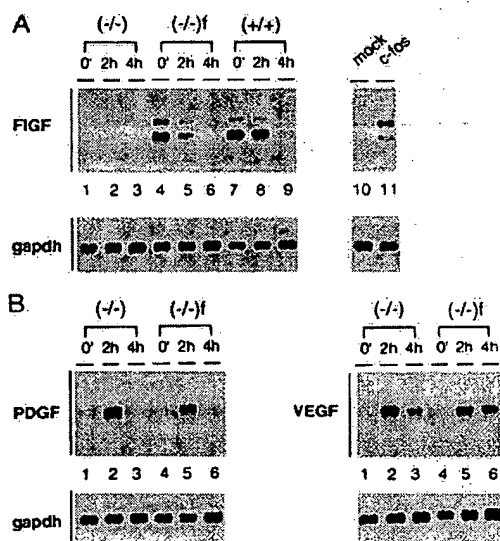


FIG. 2. (A) Expression of FIGF in cultured cells. Northern blot analysis of total RNA obtained from *c-fos* ($-/-$) fibroblasts, a cell line lacking *c-fos* (lanes 1–3); *c-fos* ($-/-$)f cells, a stable cell line expressing exogenous *c-fos*, obtained transfecting *c-fos* ($-/-$) cells with *c-fos* under the control of a constitutive promoter (lanes 4–6); wild-type *c-fos* ($+/+$) fibroblasts (lanes 7–9). Cellular RNA was extracted from cells grown for 48 h in DMEM supplemented with 0.5% FCS (time 0). The serum concentration was increased to 10% and total RNA was collected at 2 h or 4 h as indicated. Lanes 10 and 11 show FIGF expression in *c-fos* ($-/-$) fibroblasts transiently transfected with the vector alone (mock) or containing *c-fos* under the control of FBJ-LTR constitutive promoter (*c-fos*). The RNAs of the transiently transfected cells were collected 30 h after culturing the cells in DMEM containing 0.5% FCS. Each lane was loaded with 10 μ g of total cellular RNA. (B) Expression of PDGF or VEGF in cultured cells. Total cellular RNAs from *c-fos* ($-/-$) cells (lanes 1–3) or from *c-fos* ($-/-$)f cells (lanes 4–6) were extracted as indicated in A. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for RNA loading.

RESULTS

Isolation and Characterization of the FIGF cDNA. *c-fos*-deficient cells, derived from *c-fos* knockout mice (5), are defective in the induction of AP-1-responsive genes such as stromelysin and type I collagenase (14). To identify new specific *c-fos* target genes, we used the mRNA differential display technique which allowed us to isolate genes differentially expressed in *c-fos* ($-/-$) versus wild-type *c-fos* ($+/+$) cells. Few cDNA fragments, corresponding to differentially expressed mRNA, were identified and their expression pattern was confirmed by Northern blot analysis (data not shown). One of these cDNA fragments was FIGF. The full-length FIGF cDNA was isolated by screening a fibroblast cDNA library using as a probe the cDNA fragment corresponding to the 3' end of FIGF. The nucleotide sequence of the cDNA revealed a single open reading frame coding for a putative protein of 358-amino acid residues (Fig. 1A). FIGF presents a hydrophobic sequence of 20 residues at the N terminus which could code for a signal peptide (23). Comparison of the predicted FIGF protein with the SWISS-PROT data bank revealed a significant similarity of FIGF with the PDGF/VEGF family of growth factors (Fig. 1B). FIGF contains, at the same relative distance, the eight conserved cysteine residues which are characteristic of this growth factor's family (22, 24–29). These cysteine residues are involved in intra- and interchain disulfide bridges of the active dimeric molecules (30). The long N-terminal region of FIGF does not show significant similarity to known proteins. The C-terminal domain is very long and rich in cysteine residues, some of which occur in repeat units as described in the recently identified VEGF-C molecule (28).

FIGF Is Induced by *c-fos* in Cultured Fibroblasts. The expression of FIGF transcripts was examined in cells differing for the expression of *c-fos*. Northern blot analysis reveals two hybridizing FIGF transcripts of 2.4 and 4 kbp, respectively. Analysis of FIGF gene expression reveals that the FIGF messenger is barely detectable in *c-fos* ($-/-$) fibroblasts, while its expression is high in wild-type *c-fos* ($+/+$) fibroblasts (Fig. 2A, compare lanes 1 and 7). FIGF expression is completely restored in stable clones derived from *c-fos* ($-/-$) cells, expressing exogenous *c-fos* under the control of a constitutive

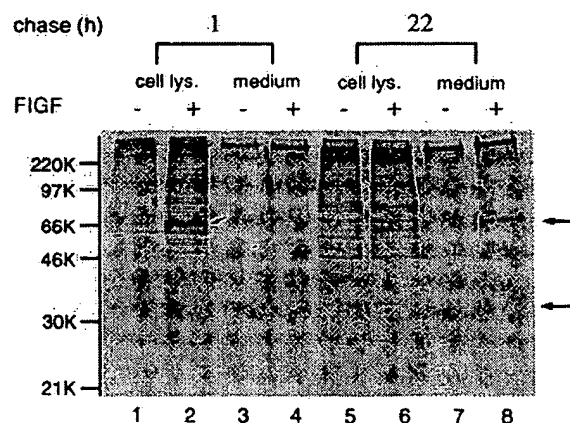


FIG. 3. Immunoprecipitation assay of the FIGF protein. COS-7 cells transiently transfected with the vector alone (–) or with a vector containing the FIGF coding sequence under the control of a CMV promoter (+) were metabolically labeled. After 1-h or 22-h chase, culture supernatants and detergent-solubilized cell lysates were subjected to immunoprecipitation and SDS/PAGE analysis under reducing conditions. Arrows indicate specific bands present only in FIGF transfected cell. The upper arrow indicates a 66-kDa protein which corresponds to the putative dimer and the lower arrow indicates a 33-kDa protein which corresponds to a putative monomer.

promoter (Fig. 2A, compare lanes 1 and 4). To exclude that in *c-fos* ($-/-$) cells the low expression is due to clonal variation, we transiently transfected these cells with *c-fos* under the control of a constitutive promoter. The transient transfection of exogenous *c-fos*, driven by the viral FBJ-LTR promoter (14), results in FIGF induction in *c-fos* ($-/-$) cells (Fig. 2A, lanes 10 and 11). These experiments show that the FIGF expression is induced by *c-fos*.

Since FIGF shows strong sequence similarities with the PDGF and VEGF, we asked whether their expression was affected by *c-fos*. As can be observed in Fig. 2B, the regulation of both PDGF and VEGF transcripts is different from that of FIGF. These growth factors are rapidly induced following serum induction and their expression is independent of *c-fos*. These data indicate that *c-fos* is required for the induction of FIGF, while the *c-fos* expression is not required for the PDGF and VEGF induction. FIGF does not differ from PDGF and VEGF in the negative regulation since they all decrease from 4 h after serum induction (Fig. 2A, lanes 6 and 9). Moreover, FIGF mRNA accumulates in quiescent cells. This pattern of expression suggests that, besides the expression of *c-fos*, additional regulatory controls are required for FIGF regulation.

FIGF Is a Secreted Protein. To verify that FIGF is a secreted protein, we transfected COS-7 cells with an expression vector containing the FIGF cDNA under the control of the CMV immediate-early gene promoter. Polyclonal antibodies, raised against recombinant FIGF produced in *E. coli*, immunoprecipitated a specific band that is observed in both the cell lysates and the conditioned media of the FIGF transfected COS-7 cells. After 1-h labeling followed by 1-h chase a specific band was mainly present in the cell lysate (Fig. 3, lane 2) while, after a chase longer than 4 h, the protein accumulated in the cell supernatant (lane 8). Under nondenaturing conditions FIGF aggregated into a multimeric form (not shown). Addition of 2-mercaptoethanol resulted in partial denaturation of the secreted protein which migrated mostly as a 66-kDa band. This corresponds to the migration of the putative dimeric form. Only a minor fraction of the secreted protein can be found at 33 kDa of molecular mass. This should correspond to the expected migration of FIGF in the monomeric form (Fig. 3, lane 8). Dimerization of FIGF could be predicted since the FIGF central domain is highly conserved and contains the eight cysteine residues involved in the dimerization of all the other known members of the PDGF and VEGF family (28–30).

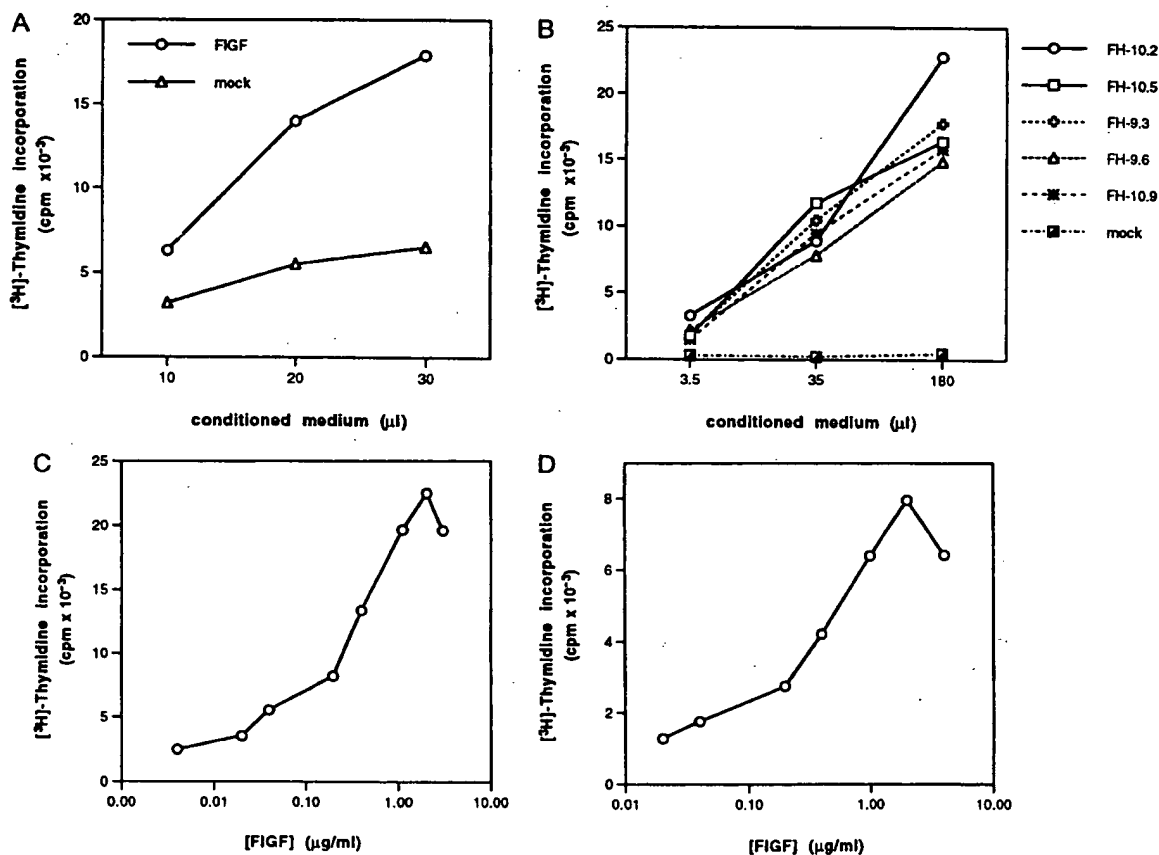


Fig. 4. FIGF induces [3H]thymidine incorporation into fibroblasts. (A) Mitogenic activity on *c-fos* ($-/-$) fibroblasts. Starved cells were stimulated with conditioned medium from COS-7 cells transiently transfected with the FIGF expression vector or with the vector alone (mock). One day after transfection COS-7 cells were split and maintained in 2% serum and conditioned media were collected after 120 h. (B) Mitogenic activity on *c-fos* ($-/-$) fibroblasts. Starved fibroblasts were stimulated with conditioned media obtained from *c-fos* ($-/-$) stable clones, named FH-10.2, FH-10.5, FH-9.3, FH-9.6, FH-10.9, and *c-fos* ($-/-$) cells (mock), constitutively expressing exogenous FIGF under the control of the CMV promoter. Culture supernatants were collected from cells maintained for 48 h in DMEM supplemented with 0.5% FCS. (C) Mitogenic activity induced by the recombinant FIGF protein on *c-fos* ($-/-$) fibroblasts. Starved cells were incubated with partially renatured recombinant FIGF. Under the same conditions, incubation with PDGF-BB (10 ng/ml; Sigma), used as a positive control, induces about 30% higher [3H]thymidine incorporation, while VEGF (10 ng/ml; Sigma) does not induce incorporation above the background (not shown). The data shown are the mean of six experiments performed with three different FIGF preparations. (D) Mitogenic activity on MEFs. Starved MEFs were stimulated with partially renatured recombinant FIGF. Under the same conditions, incubation with PDGF-BB (10 ng/ml; Sigma), used as a positive control, induces about 30% higher [3H]thymidine incorporation, while VEGF (10 ng/ml; Sigma) does not induce incorporation above the background (not shown). The data shown are the mean of six experiments performed with three different FIGF preparations. The background values were subtracted in each experiment.

FIGF Shows Mitogenic Activity on Fibroblasts. The above experiments show that FIGF is a secreted protein. We further investigated whether the conditioned medium of cells producing FIGF could promote cell growth *in vitro*, assayed as [3 H]thymidine incorporation. Conditioned medium was obtained either from transiently transfected COS-7 cells or from stable clones, obtained from *c-fos* ($-/-$) fibroblasts, expressing FIGF under the CMV promoter. The mitogenic activity of the conditioned medium containing FIGF was first tested on *c-fos* ($-/-$) fibroblasts. Both conditioned medium obtained from transfected COS-7 or constitutive FIGF expressing clones induced DNA synthesis on *c-fos* ($-/-$) fibroblasts (Fig. 4 A and B). We also tested the mitogenic activity of the recombinant FIGF protein expressed in *E. coli*. To obtain a biologically active recombinant protein, FIGF was purified from inclusion bodies and partially renatured in the presence of a mixture of reduced and oxidized glutathione. The partially refolded recombinant FIGF-induced DNA synthesis on *c-fos* ($-/-$) fibroblasts in a dose-dependent manner (Fig. 4C). As expected, *c-fos* ($-/-$) cells were also responsive to PDGF-BB, while the treatment with VEGF did not induce [3 H]thymidine incorporation (not shown). We also tested the mitogenic activity of the recombinant FIGF on mouse embryo fibroblasts. As shown in Fig. 4D, FIGF-induced DNA synthesis on these cells to levels comparable to those induced by PDGF.

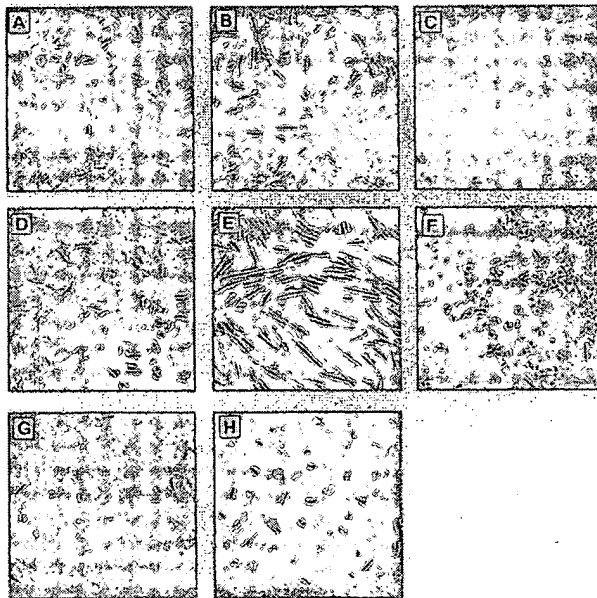


FIG. 5. (A) Morphology of *c-fos* ($-/-$) cells stably transfected with the vector alone. (B) Morphology of a cell clone derived from *c-fos* ($-/-$) cells stably transfected with the expression vector containing FIGF under the control of the CMV promoter. (C) Morphology of cells stably transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter. (D) Morphology of cells stably transfected with the expression vector containing *c-fos* under the control of the FBJ-LTR promoter. (E) A cell clone derived from the same cells as in D (expressing *c-fos* constitutively) transfected with an expression vector containing FIGF under the control of the CMV promoter. (F) A cell clone derived from the same cells as in D (expressing *c-fos* constitutively) transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter. (G) *c-fos* ($-/-$) fibroblasts cultured for 120 h in DME medium containing 0.5% serum. (H) Cells as in G but treated for 120 h with partially renatured recombinant FIGF at 2 μ g/ml. Ten independent clones obtained from three independent transfections were analyzed. All showed morphological changes similar to those observed in the figure.

FIGF Induces Morphological Alterations on Fibroblasts. The induction of transformed foci by v-H-ras, v-src, Polyoma middle T antigen, and simian virus 40 large T is not impaired in *c-fos*-deficient cells (14). Rather, *c-fos* has been implicated in tumor progression (8) and its overexpression induces a transformed cell morphology in fibroblasts and epithelial cells (6). As FIGF codes for a *c-fos*-induced growth factor, we analyzed whether its overexpression could induce a fibroblast's morphological transformation. Several independent clones overexpressing FIGF were isolated and all showed morphological alterations. Fig. 5 shows typical morphological changes observed. Cells overexpressing FIGF acquire a spindle-shaped morphology, become more refractive, and detach from the plate (Fig. 5 B versus A). The constitutive expression of *c-fos* induces morphological changes in *c-fos*-deficient cells which are similar, although less evident, to the alterations observed with the FIGF overexpression (Fig. 5D). The overexpression of both *c-fos* and FIGF leads to the same phenotype determined by FIGF overexpression and cells become extremely long (Fig. 5E). The depletion of FIGF, obtained by the expression of FIGF in the antisense orientation in *c-fos* constitutive cells, causes the complete loss of the elongated phenotype (Fig. 5F). These data show that FIGF is able to induce morphological changes on fibroblasts and suggest that FIGF is the morphological effector of *c-fos*. Cells expressing the FIGF in the antisense orientation show a slow growth rate (not shown).

To verify whether FIGF induces morphological changes on fibroblasts *in vitro* we also treated *c-fos* ($-/-$) fibroblasts with recombinant FIGF. As can be observed in Fig. 5H, that shows cells treated with recombinant FIGF for 120 h, the cell treatment with recombinant FIGF induces morphological alterations similar to the ones observed with the FIGF overexpression.

DISCUSSION

Nuclear oncogenes contribute to the cancerous state by directly altering gene regulation. The missing link between oncogenes and tumors has been the identification of oncologically relevant genes regulated by oncogenes. Some Fos target genes have already been cloned, but we are still far from understanding their role in tumor progression. To identify *c-fos*-responsive genes we isolated differentially expressed genes in cells differing for the expression of *c-fos*. Here we describe the cloning and characterization of the cDNA coding for the gene referred as FIGF. FIGF codes for a 358 amino acid residues long secreted protein. Its deduced amino acid sequence indicates that FIGF is strongly related to the PDGF/VEGF growth factors. FIGF contains in its central region the signature sequence which is characteristic for this family of growth factors. This region contains eight cysteine residues which are important for dimerization. We provide evidence that FIGF acts as a growth and morphogenic factor on fibroblasts *in vitro*. The mechanism of *c-fos* induction in response to PDGF has been well characterized (31, 32). The finding that a growth factor can be induced by *c-fos* allows to put in a consequential order of activation different growth factors of the PDGF family. Thus, the *c-fos* induction in response to PDGF or other growth stimuli may lead to the induction of other growth factors, FIGF being one of them, which most probably allows the cells to differentiate through a specific pathway.

The FIGF expression pattern analyzed by Northern blot showed a reduced expression of FIGF in *c-fos*-deficient cells. That FIGF low expression is due to the lack of *c-fos* rather than to clonal variations is demonstrated by the restoration of FIGF mRNA upon induction of exogenously expressed *c-fos* in these cells. FIGF is expressed at elevated levels, within 2 h after serum induction. This corresponds to the expression of a *c-fos*-induced gene. However, FIGF transcripts accumulates

during the quiescent phase as well. Thus, the FIGF induction is likely to require other regulatory mechanisms, probably connected with the cell cycle, in addition to the requirement for *c-fos*.

The FIGF pattern of expression differs considerably from the expression of its related genes PDGF and VEGF. These growth factors are induced rapidly after serum stimulation and their expression is not affected by *c-fos*. FIGF pattern of expression is most similar to *gas 6*. This gene, which acts as a growth factor, is abundantly expressed in serum starved cells (33, 34).

The FIGF induction by *c-fos* appears quite specific since *c-fos* cannot be substituted by other AP-1 transcription factors. In fact, in *c-fos*-deficient cells all AP-1 transcription factors except *c-fos* are normally expressed (14). In addition, the transfection of the aspecific AP-1 transcriptional activator GCN4 into these cells fails to induce FIGF (data not shown). In mammalian cells GCN4 is able to activate most AP-1 target genes, but it is nononcogenic (35). Taken together, these observations suggest that FIGF is involved in *c-fos*-dependent cell transformation. *c-fos* does not seem to be necessary for early proliferative steps of tumor formation, but it is required for malignant tumor conversion (8). Therefore FIGF could play a role in tumor progression. In this respect FIGF would not differ from VEGF. VEGF plays a role in tumor angiogenesis (36). It has recently been observed that its mRNA is elevated in papillomas originating from *c-fos* wild-type cells with respect to papillomas originating from *c-fos*-deficient cells (8). This contrasts with our results which demonstrate that, *in vitro*, the VEGF mRNA level is not affected by *c-fos*. It is likely that other events must happen before VEGF is induced during tumor progression since this effect can only be observed *in vivo*.

It has been shown that continuous *c-fos* expression induces morphological transformations *in vitro*. These morphological modifications require at least 24 h of continuous *c-fos* expression (6). We observed that FIGF is induced with a slow kinetics in response to *c-fos* and its overexpression induces morphological transformations in fibroblasts. These morphological alterations are similar to those induced by the overexpression of *c-fos* in fibroblasts. Taken together, these data suggest that FIGF is a mitogenic and morphogenic effector of *c-fos*. Thus, the role of *c-fos* in the activation of the malignant phenotype is due, at least in part, to the induction of FIGF. The involvement of FIGF in tumor progression could represent a promising step toward the therapeutic prevention of neoplastic diseases.

We thank L. D'Adamio, C. Galeotti, and M. L. Melli for the critical reading of the manuscript; B. Grandi for technical assistance; Rino Rappuoli for his precious and continuous suggestions and encouragement; and A. Tramontano for help in computer analysis. This work was supported in part by Associazione Italiana per la Ricerca sul Cancro.

- Ransone, L. J. & Verma, I. M. (1990) *Annu. Rev. Cell Biol.* **6**, 539–557.
- Angel, P. & Karin, M. (1991) *Biochim. Biophys. Acta* **1072**, 129–157.
- Karin, M. (1995) *J. Biol. Chem.* **270**, 16483–16486.
- Wang, Z.-Q., Oviatt, C., Gregoriadis, A. E., Möhle-Steinlein, U., Rüther, U. & Wagner, E. F. (1992) *Nature (London)* **360**, 742–745.
- Johnson, R. S., Spiegelman, B. M. & Papaioannou, V. E. (1992) *Cell* **71**, 577–586.
- Miao, G. G. & Curran, T. (1994) *Mol. Cell. Biol.* **14**, 4295–4310.
- Grigoriadis, A. E., Wang, Z.-Q. & Wagner, E. F. (1995) *Trends Genet.* **11**, 436–441.
- Saez, E., Rutemberg, S. E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S. H. & Spiegelman, B. M. (1995) *Cell* **82**, 721–732.
- Distel, R., Ro, H. S., Rosen, B. S., Groves, D. & Spiegelman, B. M. (1987) *Cell* **49**, 835–844.
- Schontal, A., Herrlich, P., Rahmsdorf, H. J. & Ponta, H. (1988) *Cell* **54**, 325–334.
- Kerr, L. D., Holt, J. T. & Matrisian, L. M. (1988) *Science* **242**, 1424–1427.
- Superti-Furga, G., Berges, G., Picard, D. & Busslinger, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5114–5118.
- Kho, C.-J. & Zarbl, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2200–2204.
- Hu, E., Mueller, E., Oliviero, S., Papaioannou, V., Johnson, R. & Spiegelman, B. (1994) *EMBO J.* **13**, 3094–3103.
- Chomzyski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
- Liang, P. & Pardee, A. B. (1992) *Science* **257**, 967–971.
- Liang, P., Averboukl, L. & Pardee, A. B. (1993) *Nucleic Acids Res.* **21**, 3269–3275.
- Liang, P. & Pardee, A. B. (1995) *Curr. Opin. Immunol.* **7**, 274–280.
- Bauer, D., Müller, H., Reich, J., Riedel, H., Ahrenkiel, V., Warthoe, P. & Strauss, M. (1993) *Nucleic Acids Res.* **21**, 4272–4280.
- Hoppe, J., Welch, H. A., Eichner, W. & Tatje, D. (1990) *Eur. J. Biochem.* **187**, 207–214.
- Hoppe, J., Welch, H. A. & Eichner, W. (1989) *Biochemistry* **28**, 2956–2960.
- Betsholds, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J. & Scott, J. (1986) *Nature (London)* **320**, 695–699.
- von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690.
- Leung, D. W., Cachianes, G., Kuang, W., Goeddel, D. V. & Ferrara, N. (1989) *Science* **246**, 1306–1309.
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. & Connolly, D. T. (1989) *Science* **246**, 1309–1312.
- Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. & Persico, M. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9267–9271.
- Claffey, K. P., Wilkinson, W. O. & Spiegelman, B. M. (1992) *J. Biol. Chem.* **267**, 16317–16322.
- Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lathinen, I., Kukk, E., Saksela, O., Kalkkinen, N. & Alitalo, K. (1996) *EMBO J.* **15**, 290–298.
- Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K. & Erikson, U. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 2576–2581.
- Andersson, M., Östman, A., Bäckström, G., Hellman, U., George-Nascimento, G., Westermark, B. & Heldin, C.-H. (1992) *J. Biol. Chem.* **267**, 11260–11266.
- Wagner, B. J., Hayes, T. E., Hoban, C. J. & Cochran, B. (1990) *EMBO J.* **9**, 4477–4484.
- Silvennoinen, O., Schindler, C., Schessinger, J. & Levy, D. E. (1993) *Science* **261**, 1736–1738.
- Manfioletti, G., Brancolini, C., Avanzi, G. & Schneider, G. (1993) *Mol. Cell. Biol.* **13**, 4976–4985.
- Schneider, C., King, R. M. & Philipson, L. (1988) *Cell* **54**, 787–793.
- Oliviero, S., Robinson, G., Struhl, K. & Spiegelman, B. M. (1992) *Genes Dev.* **6**, 1799–1809.
- Kim, K., Li, B., Winer, J., Armanini, M., Gillett, N., Philips, H. S. & Ferrara, N. (1993) *Nature (London)* **362**, 841–844.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.